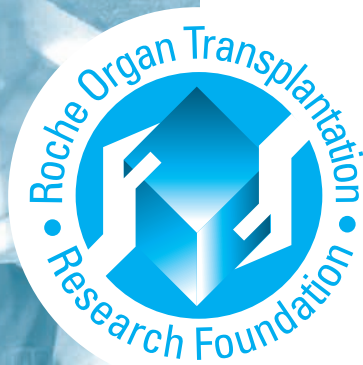


*R O T R F*

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*Roche Organ Transplantation  
Research Foundation*



# ***BIANNUAL REPORT***

*April 2001*

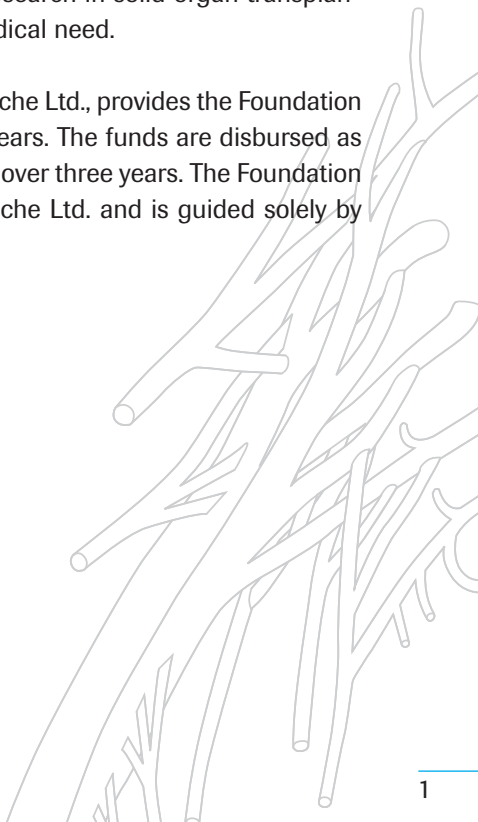


# *The Roche Organ Transplantation Research Foundation*

The mission of the Roche Organ Transplantation Research Foundation (ROTRF) is to advance the science of solid organ transplantation in order to improve the care of the thousands of patients undergoing transplantation every year. The results of the funded research projects will contribute to an understanding of many aspects of the clinical and scientific adventure of transplantation, e.g. the mechanisms of long-term organ deterioration, the consequences of tissue injury, and the opportunities to intervene in these processes.

The Foundation is an independent medical research charity that provides operating funds to established academic staff in universities, transplant centres and research institutes. The Foundation supports research in solid organ transplantation, particularly where there is an unmet medical need.

The funding, a donation from F. Hoffmann-La Roche Ltd., provides the Foundation with 25 million Swiss francs over the first five years. The funds are disbursed as grants of up to 300,000 Swiss francs distributed over three years. The Foundation is legally independent from F. Hoffmann-La Roche Ltd. and is guided solely by the Board of Trustees according to its charter.





# Table of Contents

|   |    |
|---|----|
| <b>1. Preface</b>   | 4  |
| <b>2. Facts and Figures</b>   | 5  |
| <b>3. ROTRF Grantees in Cycle V</b>   |    |
| <i>“Human Hepatic Dendritic Cells Induce Tolerance via Notch Signalling”</i><br>Prof. David Adams, Edgbaston, UK                                      | 8  |
| <i>“The Role of Mesenchymal Stem Cells in Transplantation Tolerance”</i><br>Dr. Amelia Bartholomew, Chicago, USA                                      | 9  |
| <i>“Promotion of Corneal Transplant Survival by Anti-Apoptotic Genes”</i><br>Prof. Reza Dana, Boston, USA   | 10 |
| <i>“SCID-Mouse/Human Transplant Model for Gamma Herpes Virus Infection”</i><br>Dr. Dirk Dittmer, Oklahoma City, USA                                   | 11 |
| <i>“Interaction of OX2 with its Receptor Controls Organ Rejection”</i><br>Prof. Reginald Gorczynski, Toronto, Canada                                  | 12 |
| <i>“LAT, a Molecule Critical for T Cell Activation and Function, is a Potential Substrate for Calcineurin”</i><br>Dr. Majed Hamawy, Madison, USA      | 13 |
| <i>“Chemokines and their Receptors as Therapeutic Targets to Prevent Allograft Rejection”</i><br>Dr. Matthias W. Hoffmann, Hannover, Germany          | 14 |
| <i>“The Role of TRANCE/RANK Interaction during Allogenic Immune Responses”</i><br>Dr. Régis Josien, Nantes, France                                    | 15 |
| <i>“Role of Innate Immune Recognition as Initiator of Chronic Rejection after Cardiac Transplantation”</i><br>Dr. Jörg Koglin, Munich, Germany        | 16 |
| <i>“Mechanisms of T-Cell-Mediated Injury after Renal Ischemia Reperfusion”</i><br>Dr. Hamid Rabb, Minneapolis, USA                                    | 17 |
| <i>“Donor Peripheral Blood Mononuclear Cells Homing the Thymus of Recipients to Induce Graft Tolerance”</i><br>Prof. Giuseppe Remuzzi, Bergamo, Italy | 18 |
| <i>“Prevention of Allograft Rejection by Local Expression of the IDO Gene”</i><br>Dr. Peter Terness, Heidelberg, Germany                              | 19 |
| <i>“Mechanisms Involved in Prevention of Donor-Specific Cardiac Allograft Rejection by Regulatory T Cells”</i><br>Dr. Li Zhang, Toronto, Canada       | 20 |

#### 4. Progress Reports of ROTRF Grantees

|  |    |
|--|----|
| <i>“The Role of Oxidative Stress Post-Hyperacute Xenograft Rejection”</i><br>Prof. Anthony J.F. d’Apice, Melbourne, Australia – Grantee in Cycle I                                 | 21 |
| <i>“Gene Therapy-Based Treatment for Insulin-Dependent Diabetes Mellitus”</i><br>Dr. Tausif Alam, Madison, USA – Grantee in Cycle III  | 25 |
| <i>“Molecular Regulation of Liver Cell Transplantation”</i><br>Dr. Jorge A. Bezerra, Cincinnati, USA – Grantee in Cycle I  | 28 |
| <i>“Identification of Novel Porcine Immunoregulatory Molecules”</i><br>Prof. Alfred L.M. Bothwell, New Haven, USA – Grantee in Cycle I   | 31 |
| <i>“Study of the Role of Gamma-Delta T Lymphocytes in the Immune Response Directed against Human Cytomegalovirus”</i><br>Dr. Julie Dechanet, Bordeaux, France – Grantee in Cycle I | 34 |
| <i>“The Role of Senescence and Telomere Shortening in Chronic Rejection”</i><br>Prof. Philip F. Halloran, Edmonton, Canada – Grantee in Cycle I                                    | 38 |
| <i>“Identification of a Novel Protein Involved in Rejection of Transplanted Organs”</i><br>Dr. Robert A. Kirken, Houston, USA – Grantee in Cycle III                               | 41 |
| <i>“Effect of T Cell Costimulatory Blockade with Bone Marrow Transplantation on Organ Transplant Rejection”</i><br>Dr. Kenneth A. Newell, Atlanta, USA – Grantee in Cycle III      | 44 |
| <i>“Derivation of Insulin-Producing Cells from Embryonic Stem Cell Lines”</i><br>Dr. Jon S. Odorico, Madison, USA – Grantee from Cycle I   | 47 |
| <i>“Role of Viral Chemokine Receptors in Cytomegalovirus–Accelerated Transplant Vascular Sclerosis”</i><br>Dr. Susan L. Orloff, Portland, USA – Grantee in Cycle III               | 50 |
| <i>“Heme Oxygenase-1: An Anti-Inflammatory Molecule that Promotes Organ Graft Survival”</i><br>Dr. Miguel P. Soares, Boston, USA – Grantee in Cycle I                              | 53 |
| <i>“Cytokine-Activated Signalling in T Cells is Required for Tolerance Induction by Allochimeric Protein”</i><br>Dr. Stanislaw M. Stepkowski, Houston, USA – Grantee in Cycle III  | 56 |
| <i>“Studies of a Novel Dendritic Cell (DC) Population in Organ Allograft Survival”</i><br>Prof. Angus Thomson, Pittsburgh, USA – Grantee in Cycle I                                | 59 |
| <i>“Gene Expression of Tolerance-Mediating Allospecific Cells”</i><br>Dr. Hans-Dieter Volk, Berlin, Germany – Grantee in Cycle I   | 62 |



## 1. Preface

On behalf of the Board of Trustees, I am pleased to announce that thirteen research grants have been awarded to scientists around the world following the fifth cycle of grant review. A total of 2 million Swiss Francs (CHF) has been awarded in this funding cycle.

The ROTRF was established by the Roche Group in 1998 as a charitable, autonomous and legally independent organisation. At that time, the Roche Group indicated its commitment to the international transplant community by providing funding of CHF 5 million annually for 5 years from inception to a total of CHF 25 million. The Foundation has two funding cycles per year and therefore the original funding from the Roche Group will cover ten funding cycles. Having just completed the fifth funding cycle, half of the original available funding has now been allocated. In this relatively short time, the ROTRF has firmly established itself as a unique grant-awarding body in the field of solid organ transplantation. In each cycle, many letters of intent are submitted from established scientists and clinicians from around the world, though principally from North America and Europe. The Trustees and the Scientific Advisory Committee have in general been very impressed with the level of scientific quality and originality shown by the applicants.

The Progress Reports published in this and previous Biannual Reports demonstrate the quality of work that has been carried out on ROTRF grants. Many high-quality papers have already been published in respected international journals, and it is to be expected that the number of papers will grow considerably over the next few years.

Thus ROTRF is clearly fulfilling its mission to advance the science of solid organ transplantation.

Special credit goes to the investigators who have come to us with their projects, and to the outstanding work of the scientific advisory board who have ranked the letters of intent and the full-length applications. This is a unique process – an international competition with an international review, and much of the credit for the success goes to these individuals.

Finally, as we pass the midpoint of our mandate, we thank the Roche Group for their generosity in making the ROTRF possible. The transplant community has benefited and is grateful for this special gift.

On behalf of the Board of Trustees

Phil Halloran



## 2. Facts and Figures

### Funding Cycle V – Letter of Intent Submission in October 2000

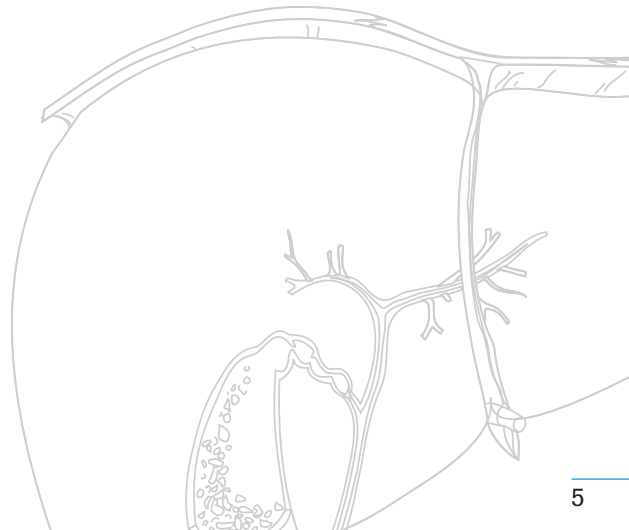
In the fifth ROTRF funding cycle, 119 letters of intent were received from scientists around the world. Almost half of the applications came from the United States (45%), while 38% came from Europe, the major countries being Germany (13%) and the UK (12%). In total, North America accounted for 49% of the applicants, and Asia, Oceania and South America together accounted for the remaining 13% of the applicants.

The Scientific Advisory Committee of the ROTRF evaluated all the applications, which were submitted electronically via the ROTRF homepage ([www.ROTRF.org](http://www.ROTRF.org)), on the basis of originality and scientific excellence. The top 21 applications were invited to submit full paper applications, and the 20 applications received subsequently underwent a second thorough review by the Scientific Advisory Committee and the Board of Trustees.

ROTRF Grants were finally awarded to 13 applicants: five from the USA; three from Germany; two from Canada; and one each from France, Italy and the UK (see the blue dots on the world map on the following page). Only two of the Grantees in funding cycle V are female.

The abstracts of the novel and promising research projects that received funding in cycle V are presented on the following pages. Their research interests focus mainly on the improvement of long-term graft survival and prevention of chronic organ dysfunction, induction of tolerance, the development of new immunosuppressive agents, and the improvement of donor organ preservation.

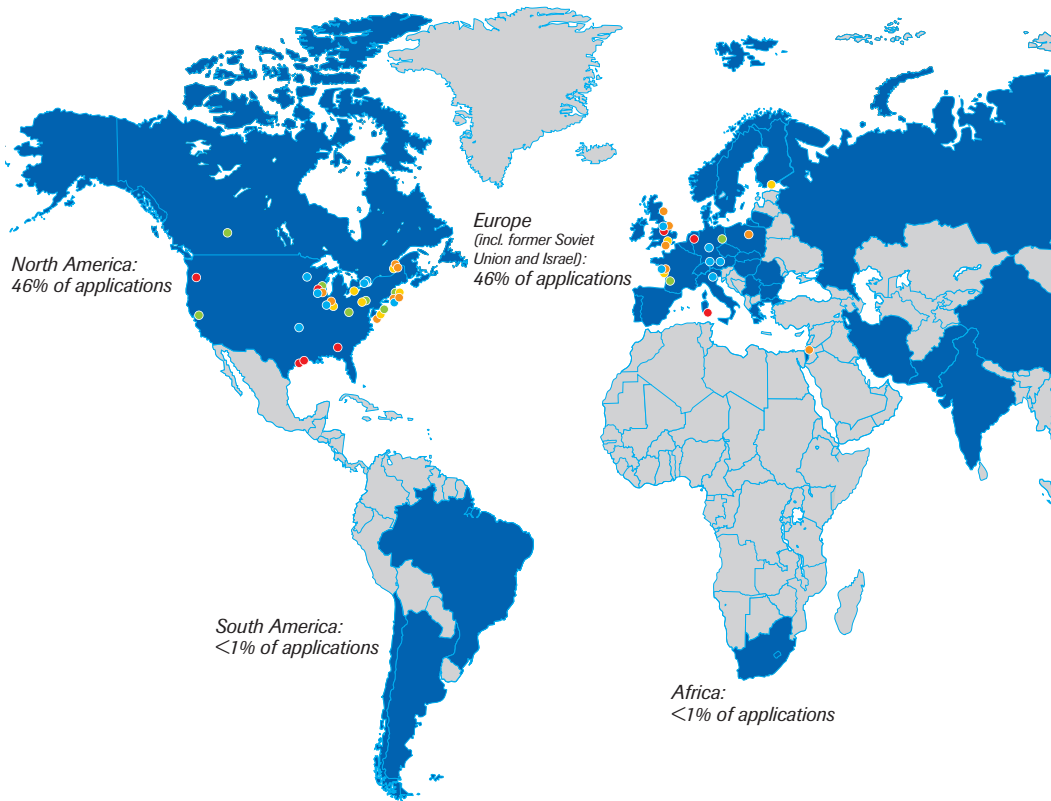
In this fifth cycle of ROTRF Grant Awards, a total of 2.0 million Swiss Francs was allocated.

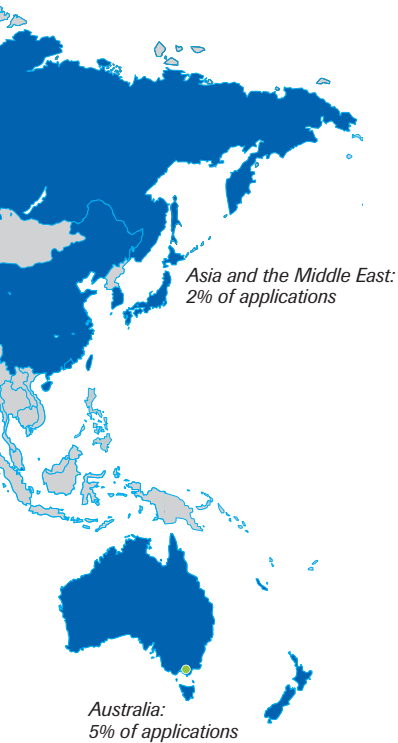




# The Global View of Applications to the ROTRF

## Distribution of ROTRF applications worldwide





## Cycle I Grantees

Berlin, Germany  
Bordeaux, France  
Boston, USA  
Cincinnati, USA  
Edmonton, Canada  
Madison, USA  
Melbourne, Australia  
New Haven, USA  
Pittsburgh, USA  
San Francisco, USA

## Cycle II Grantees

Boston, USA  
Chicago, USA  
Helsinki, Finland  
London, Canada  
Montreal, Canada  
Nantes, France  
New York, USA  
Oxford, UK  
Pittsburgh, USA

## Cycle III Grantees

Atlanta, USA  
Birmingham, UK  
Cagliari, Italy  
Houston, USA  
Houston, USA  
Madison, USA  
Nijmegen, The Netherlands  
Portland, USA  
Winnipeg, Canada

## Cycle IV Grantees

Boston, USA  
Boston, USA  
Chicago, USA  
Dundee, UK  
Laval, Canada  
Madison, USA  
Montreal, Canada  
Nantes, France  
Newcastle-upon-Tyne, UK  
Oxford, UK  
Philadelphia, USA  
Rehovot, Israel  
Warsaw, Poland

## Cycle V Grantees

Bergamo, Italy  
Boston, USA  
Chicago, USA  
Edgbaston, UK  
Hanover, Germany  
Heidelberg, Germany  
Madison, USA  
Minneapolis, USA  
Munich, Germany  
Nantes, France  
Oklahoma City, USA  
Toronto, Canada  
Toronto, Canada





### 3. Grant Awards in Cycle V

**Prof. David Adams, Principal Investigator**

*Dr. Sarah Goddard, Co-Applicant*



**University of Birmingham, Edgbaston, UK**

#### **Human Hepatic Dendritic Cells Induce Tolerance via Notch Signalling**

Although liver transplantation is increasingly successful, patients need to take powerful drugs for the rest of their lives to prevent rejection. These drugs have serious side-effects and quality of life would be greatly enhanced if their use could be avoided or minimised. One way to do this would be to develop a treatment that allowed the transplant to be accepted by the patient without the need for long-term anti-rejection drugs, a phenomenon called immunological tolerance. There is evidence that the liver may be more amenable to such treatment than other organs because in some animals livers can be transplanted without any anti-rejection drugs and in humans liver transplants require less anti-rejection therapy than heart or kidney transplants. The underlying mechanisms of this tolerance are not understood.

We shall investigate the role of important regulatory cells – dendritic cells (DCs) – in this process. DCs have unique properties that allow them to regulate immune responses by switching on or off lymphocytes, the cells that cause transplant rejection. We have shown that DCs from human liver differ when compared with those from other sites, such as the skin (skin transplants are rapidly rejected). We now want to find out whether these differences allow the liver DCs to switch off rejection and, if so, how they do this.

We have found that human liver DCs express much greater amounts of a molecule called Jagged when compared with skin DCs. Jagged binds to a receptor called Notch on other cells and this interaction can affect the fate of the cell and prevent it from becoming activated. Because Notch is present on lymphocytes, we propose that Jagged on liver DCs will be able to trigger notch and turn the potentially harmful lymphocytes into regulatory cells that prevent rejection from developing. If this new hypothesis is correct, it may be possible to manipulate the Jagged/Notch system to increase the chance of transplants being accepted without the need for anti-rejection drugs.

**Dr. Amelia Bartholomew, Principal Investigator**

*Dr. Pamela Witte, Research Associate*

*Dr. Anita Chong, Research Associate*

*Ms. Mandy Siatskas, Research Associate*



**University of Illinois at Chicago, Chicago, USA**

**The Role of Mesenchymal Stem Cells in Transplantation Tolerance**

The induction of transplantation tolerance remains a foreseeable yet elusive goal in transplantation. Experimental approaches using donor bone marrow cells to eliminate recipient reactivity against donor organs are promising, but presently limited in appeal due to the need for rigorous recipient conditioning regimens. High doses of bone marrow stem cells can induce tolerance with less toxic conditioning regimens.

The success of this approach may be in part due to the increased frequency of other immunologically active cell types. Mesenchymal stem cells (MSCs) are multipotential cells that can be induced to differentiate into elements of the bone marrow microenvironment, such as bone cells, adipocytes, and stromal cells. Stromal progeny of MSCs have been implicated in regulatory signals that inhibit or promote lympho- and myelopoiesis, differentiation, and proliferation *in vitro*. When an extensive complement of MSC progeny, via bone fragments, is transplanted along with hematopoietic stem cells (HSCs), increased hematopoietic engraftment and transplantation tolerance have been observed. We have shown that transplantation of the bone marrow microenvironment without HSCs can lead to the permanent acceptance of murine cardiac allografts. Further, MSCs inhibit T cell proliferation *in vitro*, prolong skin graft survival, and home to the bone marrow compartment, thereby potentially influencing the host microenvironment. These observations have led us to hypothesize that MSCs have immunomodulatory properties and play a major role in the induction of transplantation tolerance.

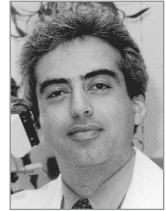
In these studies, we will test whether MSCs directly affect the induction of tolerance by engrafting them within the thymus and altering the recipient T cell repertoire. We will also test whether the MSCs play an indirect role in the induction of tolerance through the facilitation of HSC engraftment either with or without host conditioning. Insights gained on the role donor MSCs play in allograft acceptance may then be applied to our pre-clinical model for the development of novel pre-clinical cellular therapies in transplantation tolerance.

**Prof. Reza Dana, Principal Investigator**

*Prof. Andrius Kazlauskas, Co-Applicant*

*Dr. Ying Qian, Research Assistant*

*Mr. Qiang Zhang, Research Technician*



**Schepens Eye Research Institute, Boston, USA**

**Promotion of Corneal Transplant Survival by Anti-Apoptotic Genes**

Corneal transplantation is by far the most common form of tissue transplantation. While many grafts have a generally favorable prognosis under topical immune suppression, the medications used to prevent graft failure are toxic to the eye, commonly causing cataracts and glaucoma. In addition, many corneal grafts performed in inflamed host beds are deemed high-risk by virtue of their dismal prognosis due to rejection rates that far exceed 50%.

Corneal clarity is critically dependent upon normal functioning of a monolayer of cells known as the corneal endothelium. These cells are responsible for the pump function that keeps the cornea free from swelling. Almost all forms of corneal transplant failure are due to endothelial cell dysfunction or death. Moreover, many endothelial cells are lost in the process of eye banking prior to transplantation, so that the grafted donor tissue becomes particularly vulnerable to failure if its endothelium comes under immune attack.

Corneal endothelial cell death is mediated by a process known as apoptosis. Many of the genes responsible for apoptosis have been defined, as have genes that downregulate this process – known as anti-apoptotic genes. Gene therapy is the strategy by which selective genes are either activated or downregulated. Our preliminary work shows great promise in using a viral vector to selectively infect corneal endothelial cells with the desired genes after organ procurement but before transplantation. We propose to use this strategy to overexpress anti-apoptotic genes in the endothelium so as to render grafted corneas resistant to a variety of stresses, including those that lead to cell death during storage or immune-mediated cell death after transplantation.

We hope that successful completion of our proposal will lead to significant enhancement of graft survival, even in the adverse setting of high-risk transplantation, as well as better organ preservation over longer periods.

**Dr. Dirk Dittmer, Principal Investigator**



**University of Oklahoma Health Sciences Center,  
Oklahoma City, USA**

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### **SCID-Mouse/Human Transplant Model for Gamma Herpes Virus Infection**

The purpose of this proposal is to develop a small animal model for human herpes virus infections. Normally, herpes viruses, such as Epstein-Barr virus or Kaposi's sarcoma-associated herpes virus, cause no more than fever, but in organ transplantation patients these viruses grow out of control and cause death. Since these viruses only grow in human tissues, we decided to transplant human cells into mice. We can thus infect the mice with the human virus and study the viral biology. We can also test anti-viral drugs or drugs that are used in organ transplantations for their effect on herpes virus replication.



**Prof. Reginald Gorczynski, Principal Investigator**

*Dr. Gary Levy, Co-Applicant*



**University Health Network, Toronto, Canada**

### **Interaction of OX2 with its Receptor Controls Organ Rejection**

Induction of immunological tolerance is important for promoting long-term organ and/or tissue graft acceptance. Our investigations have shown that tolerance to renal allografts in mice/rats followed after infusion of donor cells into the liver (via the portal vein), and that this was associated with increased expression by recipient cells of a novel molecule, OX2, which was crucial for tolerance induction. As “proof of principle” for this hypothesis, we showed that antibodies to OX2 blocked tolerance to renal allografts, while a soluble form of OX2 could induce tolerance in rodents. Our institution is currently engaged in testing these reagents in a large animal transplant model, with a view to phase 1 clinical trials in the future.

The biochemistry of OX2 suggested that it only delivers a tolerance signal following engagement of its receptor (OX2R). We have prepared antibodies to OX2R, and in addition are making a soluble form of OX2R. We propose to test the hypothesis that OX2R itself provides a key signal for tolerance induction in transplantation by using anti-OX2R to induce tolerance (and soluble OX2R to block that induction).

Other experiments in our laboratory are involved in the generation of transgenic mice that overexpress the OX2 molecule on all tissues (OX2tg/tg), and mice in which the gene encoding the receptor has been genetically deleted (OX2R KO). A group at DNAX Research Institute has already created an OX2 KO mouse, and showed that it has susceptibility to autoimmune disease. We predict that transplants will be unsuccessful in OX2 KO mice, unless organs are used from OX2tg/tg. Transplants in OX2R KO mice will not be accepted, even from OX2tg/tg mice.

These studies from animal transplant models will provide information on immunoregulation applicable to numerous human diseases, including transplantation, allergy and autoimmunity.

**Dr. Majed Hamawy, Principal Investigator**

*Dr. Zheng Chang, Research Associate*



**University of Wisconsin Medical School, Madison, USA**

### **LAT, a Molecule Critical for T Cell Activation and Function, is a Potential Substrate for Calcineurin**

Blocking the activation of the serine/threonine phosphatase calcineurin in T cells by CsA and FK506 has been useful for preventing allograft rejection in humans. Yet blocking calcineurin activation by CsA and FK506 has also been associated with side effects that limit the clinical use of these drugs. Although calcineurin inhibition blocks IL-2 production, it increases the release of cytokines such as TGF- $\beta$  from T cells. Also, because calcineurin is found in almost all cells, blocking its activation does not only affect T cell function but also disrupts the function of other cells.

Given that blocking calcineurin signaling pathways has proven useful for blocking T cell activation, we initiated studies to define T-cell-specific targets for calcineurin that are important for T cell activation. The identification of these targets should be useful for designing drugs that block the activation of such targets and in turn specifically block T cell function with no or minimal side effects. We have shown that blocking calcineurin activation with CsA and FK506 increases the expression and reduces the electrophoretic mobility of LAT, a signaling molecule found predominantly in T cells that is critical for T cell activation and development. Incubation of purified calcineurin with LAT *in vitro* led to dephosphorylation of LAT and an increase in its electrophoretic mobility on SDS-PAGE, suggesting that LAT is a potential substrate for calcineurin. Thus, CsA and FK506, by blocking calcineurin activation, appear to regulate both the cellular level and the phosphorylation level of LAT in T cells.

We aim to identify the amino acid residues in LAT whose phosphorylation is regulated by calcineurin inhibitors, and to examine the role of these residues in T cell function. Because LAT is predominantly found in T cells, the data obtained could provide a means to design new drugs that specifically block T cell activation and prevent allograft rejection with minimal or no side effects.

**Dr. Matthias W. Hoffmann, Principal Investigator**

*Prof. Reinhold Förster, Co-Applicant*



**Medizinische Hochschule Hannover,  
Hannover, Germany**

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### **Chemokines and their Receptors as Therapeutic Targets to Prevent Allograft Rejection**

T lymphocytes play a central role in the rejection of organ allografts. To be activated, antigen-unexperienced (naive) T lymphocytes migrate to secondary lymphoid organs, such as lymph nodes, the spleen, Peyer's patches, or tonsils. There, naive T lymphocytes are activated by antigen-presenting cells that carry foreign antigens from the graft to these secondary lymphoid organs. Since migration of naive T lymphocytes to secondary lymphoid organs critically depends on chemotactic lymphokines (chemokines), one strategy to block T lymphocyte activation consists of the blockade of these chemokines or their receptors.

The chemokine receptor required for migration of naive T lymphocytes into secondary lymphoid organs is CCR7. Therefore, the main topic of the research proposal will be to study to what extent allogeneic skin and heart grafts will be accepted in CCR7-deficient mice, and how this is effected. These results will be used to develop therapeutic strategies to prevent the rejection of organ allografts at a very early point in T cell activation. This treatment strategy would spare the immunosuppression of beneficial antigen-experienced (memory) T lymphocytes and would maintain their anti-bacterial, anti-viral or anti-tumour activities.

In summary, this research proposal aims to develop a novel type of immunosuppression that specifically targets the organ allograft, without suppressing beneficial T cells reactive to pathogens or tumour cells.

**Dr. Régis Josien, Principal Investigator**

*Dr. Maria-Cristina Cuturi, Co-Applicant*

*Dr. Ignacio Anegon, Research Associate*

*Cédric Lovet, Research Associate*

*Cécile Voisine, Research Associate*

*Jean-Marie Heslan, Research Associate*

*Dr. Yongwon Choi, Consultant*



**INSERM, Nantes, France**

**The Role of TRANCE/RANK Interaction during Allogenic Immune Responses**

Understanding immune mechanisms that lead to acute and chronic allograft rejection is an important step towards identification of molecular targets of current and new immunosuppressive drugs. Recent results have shown that the CD40L molecule, which is expressed on activated T cells, and its counter receptor CD40, expressed on antigen-presenting cells (dendritic cells, macrophages and B lymphocytes), represent a major pathway for T cell activation. CD40L blockade has been shown to delay allograft rejection in rodents and monkeys and will probably be used in the future in human organ transplantation.

Recently, a new member of the tumour necrosis factor (TNF) family, called TRANCE (TNF-related activation-induced cytokine) has been described. This molecule shares functional properties with CD40L but appears to be more specialised in the interaction between T cells and dendritic cells, two subsets that play a pivotal role in immune responses to allografts. *In vivo* studies indicate that, in the absence of the CD40L pathway, the immune system can use the TRANCE pathway to activate T helper cells.

In this proposal, we will analyse the role of TRANCE/RANK interactions during immune responses leading to acute and chronic allograft rejection using rat models. The expression of TRANCE and its receptor RANK will be assessed in allografts and lymphoid organs in different experimental settings. TRANCE blocking reagents will be injected into allograft recipients to determine the role of this pathway in experimental acute and chronic allograft rejection. The manipulation of this system will include gene transfer experiments using recombinant adenoviruses. We will also analyse immune responses to allografts in TRANCE-deficient mice. Based on these studies and their results, we will design new experiments to test the effects of TRANCE blockade in a primate model of renal allograft.



**Dr. Jörg Koglin, Principal Investigator**

*Prof. Mary E. Russell, Consultant*



**University of Munich, Munich, Germany**

### **Role of Innate Immune Recognition as Initiator of Chronic Rejection after Cardiac Transplantation**

Even with optimised immunosuppressive strategies, chronic rejection with the development of an accelerated form of arteriosclerosis remains the major limitation of long-term graft survival after cardiac transplantation. While acquired immune responses have been identified to propagate this form of rejection, the proximal events leading to their activation remain unknown. Recently, evolutionarily ancient innate immune recognition has been found to control the activation of adaptive immune responses in antibacterial processes. As proximal signalling events, specific molecular patterns are recognised by members of the Toll-like receptor family expressed on antigen-presenting cells.

Activation of the human homologues of this receptor family has been shown to induce the expression of a variety of cytokines and costimulatory molecules known to be crucial for activation of adaptive immune responses in chronic rejection. However, while these innate mechanisms are well characterised in the cellular defence against infectious agents, their role in chronic cardiac rejection remains unknown. We hypothesise that innate immune responses play an essential role in the initiation and control of the immune cascade responsible for the chronic rejection of transplanted hearts.

To validate this hypothesis, this project aims to characterise Toll-receptor signalling in monocytes from cardiac transplant recipients with and without chronic rejection, to compare costimulatory signalling and cytokine expression in these patients, and to identify potential specific molecular patterns responsible for the activation of innate immunity. Proof of the proposed hypothesis would not only open new ways to stratify the individual risk of transplant recipients to develop cardiac allograft vasculopathy (CAV), but would also point towards novel strategies to interrupt innate immune recognition in order to prevent chronic rejection after cardiac transplantation.

**Dr. Hamid Rabb, Principal Investigator**



**Minneapolis Medical Research Foundation,  
Minneapolis, USA**

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### **Mechanisms of T-Cell-Mediated Injury after Renal Ischemia Reperfusion**

Kidney ischemia is the main cause of the initial poor function of an organ transplant. Kidney ischemia predisposes to acute rejection, which in turn predisposes to chronic rejection – the major long-term cause of graft loss. There is no specific treatment for kidney ischemia. Recently, we and others have identified that the circulating T cell is an important mediator of kidney ischemic damage. This was somewhat of a surprise finding, as the T cell is known to be important in rejection, but not in organ procurement injury.

The observation that the T cell is a mediator of kidney ischemia has been well verified in experimental models. However, the underlying mechanisms for this are unknown. We therefore propose to begin to elucidate these underlying mechanisms. Based on our preliminary data using a mouse model of kidney ischemia, we hypothesize that a major subset of T cells, called Th1, functionally mediates T-cell-mediated kidney injury, and that T-cell-mediated injury requires engagement of a key receptor on the surface of the kidney.

We plan to test this hypothesis using an established mouse model of kidney ischemia, various strains of generated mutant mice, sensitive measures of kidney function, and molecular and cellular detection techniques. Our results will have implications for the understanding of organ procurement injury and may help lead to new therapies. In addition, the results will also extend and challenge our basic understanding of the immune system.

**Prof. Giuseppe Remuzzi, Principal Investigator**

*Dr. Ariela Benigni, Co-Applicant*

*Dr. Marina Noris, Co-Applicant*



**Mario Negri Institute for Pharmacological Research,  
Bergamo, Italy**

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**Donor Peripheral Blood Mononuclear Cells Homing  
the Thymus of Recipients to Induce Graft Tolerance**

The success of clinical organ or tissue transplantation currently depends on the use of non-specific immunosuppressive drugs. However, long-term immunosuppression increases the risk of life-threatening infections and cancer in transplant recipients, and patients receiving immunosuppressive drugs still experience chronic graft rejection. To eliminate or reduce the need for long-term immunosuppressive drugs, a strategy would be to reprogram the host's immune system to selectively ignore, or tolerate, the transplant without compromising protection against pathogens. This would allow the induction of donor antigen-specific immunologic unresponsiveness.

In an effort to find strategies less toxic than current conditioning protocols to induce transplantation tolerance, we found that pretransplant donor peripheral blood mononuclear cell (PBMC) infusion allowed indefinite kidney allograft survival in rats. Graft tolerance correlated with the presence of donor cells in the host thymus (microchimerism). These findings provide the basis for exploring the possibility that enhancing migration of donor cells into the host thymus would favour the development of donor-specific transplantation tolerance. We hypothesised that this could be accomplished by transfecting donor cells with genes encoding molecules, such as CD44 and/or CCR9, that are critical for cell migration into lymphoid organs, including the thymus.

Since dendritic cells (DCs) represent the ideal donor cells for transfection, in that under naive conditions they express variable levels of CD44 and very low levels of CCR9 molecules on their surface, and given the fact that we previously found that induction of graft tolerance occurs through migration of donor cells into the host thymus, donor transfected DCs will be used to test our hypothesis. From this series of studies in rodent models of kidney and heart transplantation we expect to document that donor DCs transduced with CD44 and/or CCR9 genes allow the development of graft tolerance by homing to the host thymus where they activate donor-specific tolerogenic pathways.

**Dr. Peter Terness, Principal Investigator**



**University of Heidelberg, Heidelberg, Germany**

### **Prevention of Allograft Rejection by Local Expression of the IDO Gene**

When a foreign tissue or organ is transplanted into an unrelated person the immune system of the recipient destroys the graft. This process is known as the rejection reaction. To suppress the rejection, patients carrying transplanted organs receive immunosuppressive drugs. These drugs, however, not only suppress the unwanted immune reaction to the foreign tissue but also that to harmful aggressors, such as bacteria, viruses, etc., which often leads to serious infections and other diseases. The ultimate objective of transplantation immunology is to specifically inhibit the response against the graft while leaving the remaining immune defence unaltered.

Nature has provided us with at least one example of how this can be accomplished. Pregnant women carry foetuses, half of whose genes come from the mother and the other half from the father. The part inherited from the father is foreign to the mother. Nevertheless, the expectant mother does not reject the foetus. It is the placenta, the organ located between the mother and the conceptus, which is responsible for suppressing the lymphocyte attack.

Recently, a gene active in placental cells that suppresses the activity of these lymphocytes has been identified. In the current project we plan to insert this gene into the prospective graft. In order to test the action of the gene, a rat heart transplant model has been chosen. It is expected that after expression of the suppressive gene in the transplanted heart the infiltrating lymphocytes will be inactivated. After successful completion of animal experiments, first clinical trials will be considered.

**Dr. Li Zhang, Principal Investigator**

*Dr. Myron Cybulsky, Co-Applicant*



**University Health Network, Toronto, Canada**

### **Mechanisms Involved in Prevention of Donor-Specific Cardiac Allograft Rejection by Regulatory T Cells**

Organ transplantation is a potent therapeutic modality for patients with end-stage organ failure. While it is generally successful, there are still a number of significant problems that affect all transplant patients. Most graft recipients have to take multiple immunosuppressive medications for as long as the transplant is functioning. These medications are expensive and have toxic side effects. Transplant patients also have a high incidence of opportunistic infections and malignancies due to suppression of the immune system of recipients by non-specific immunosuppressive drugs. Moreover, use these drugs does not ensure permanent survival of the transplanted organs. Most transplanted organs are eventually rejected. The major clinical goal of organ transplantation is to induce long-term graft survival without the need for non-specific toxic immunosuppressive drugs.

Recent work from our group demonstrated that exposure of recipient to donor white blood cells under appropriate conditions leads to activation and expansion of recipient regulatory T cells. We have further demonstrated in mice that these activated regulatory T cells can migrate to transplanted organs and tissues, specifically inhibit anti-graft immune responses, and enhance donor-specific skin graft survival.

In this proposal, we will investigate how to effectively activate recipient regulatory T cells to prevent cardiac graft rejection in mouse models. We will delineate the mechanisms involved in regulatory T cell migration, and antigen-specific suppression. We will also determine whether regulatory T cells can be identified and activated in humans. The results from these studies will enhance our understanding of the mechanisms of antigen-specific suppression. They will also provide a basis for a trial using regulatory T cells for the prevention of allograft rejection and the treatment of autoimmune diseases.



## 4. Progress Reports

**Prof. Anthony J. F. d'Apice, Principle Investigator**

*Dr. Peter J. Cowan, Co-Applicant*

*Dr. Trixie A. Shinkel, Associate*



**St. Vincent's Hospital Melbourne, Melbourne, Australia**

### **The Role of Oxidative Stress in Post-Hyperacute Xenograft Rejection**

Xenotransplantation is viewed as a potential solution to the chronic shortage of human organs for transplantation. With the availability of effective strategies to prevent hyperacute rejection of pig-to-primate xenografts, acute vascular rejection (AVR) is regarded as the current barrier to be overcome. Exactly what initiates AVR is unclear, but the key event appears to be injury to and activation of graft endothelial cells (EC) by multiple factors including reperfusion, binding of xenoreactive antibodies and complement, and interactions with activated platelets and infiltrating leukocytes. Ischemia reperfusion (IR) injury causes oxidative-stress-induced activation of graft ECs resulting in a cascade of cytokine and adhesion molecule upregulation. The destructive agents in IR injury are reactive oxygen intermediates (ROIs). In addition, the predominant cell type infiltrating vascularised xenografts, namely macrophages, also generate ROIs, thus contributing further to the injury and activation of graft ECs.

ROIs cause tissue injury and destruction by damaging DNA, proteins and cell membranes. Under normal physiological conditions, cells maintain an equilibrium between oxidant and antioxidant mechanisms by the action of antioxidant enzymes, such as superoxide dismutase, catalase and glutathione peroxidase. However, in situations such as AVR, the magnitude of ROIs generated at the surface of and within the xenograft endothelium may overwhelm the endogenous antioxidant enzymes, resulting ultimately in loss of graft function. The hypothesis of this application is that the transgenic expression of a "balanced combination" of antioxidant enzymes in the endothelium of a xenograft will afford protection from ROI-induced xenograft injury and will prolong xenograft survival.

## Aims

1. To prepare expression constructs for native and epitope-tagged versions of copper zinc superoxide dismutase (CuZnSOD), glycosylphosphatidylinositol-linked extracellular superoxide dismutase (GPI-linked ECSOD) and glutathione peroxidase (GPx) and to establish expression and activity of the enzymes *in vitro*.
2. To generate transgenic mice expressing combinations of the above enzymes and to show expression and activity of the enzymes *in vivo*.
3. To determine the functional effect of antioxidant enzyme overexpression on AVR using the established Gal knockout mouse heart transplant model.

## Results

### **Transfection and *in vitro* testing**

In the first year of this project, constructs for native and epitope-tagged forms of CuZnSOD, GPI-linked ECSOD and GPx were generated and enzyme activity assays were established. Preliminary results for the expression and activity of both CuZnSOD and GPI-linked ECSOD were reported. All other work mentioned in this report has been carried out in the second year of this project.

Native and epitope-tagged forms of CuZnSOD and GPI-linked ECSOD have been transiently transfected into COS cells and expression detected by flow cytometric analysis (FACS) of intact (GPI-linked ECSOD) or saponin-permeabilised (CuZnSOD) cells using specific antibodies to the antioxidant enzyme and/or the epitope tag. High levels of human CuZnSOD and ECSOD expression were detected. Comparable levels of expression were observed with native and epitope-tagged forms of the enzymes.

Transient transfection of native and epitope-tagged forms of GPx was performed. However, antibodies to GPx or the epitope tag were unsuccessful in detecting expression of GPx by either FACS or western blotting.

SOD activity in lysates from transfected cells was measured spectrophotometrically by monitoring the inhibition of nitrite formation from hydroxylammonium chloride. Native and epitope-tagged forms of CuZnSOD and GPI-linked ECSOD showed increases in enzyme activity compared with vector-transfected cells ranging between 145 and 158%.

GPx activity in lysates from transfected cells was also measured spectrophotometrically using glutathione reductase and NADPH in a coupled assay procedure. An increase in enzyme activity of 270% compared with vector-transfected cells was observed for both the native and epitope-tagged form of GPx.

## Generation and analysis of transgenic mice

Transgenic mice (single and triple combinations) were generated using a generic microinjection construct comprising the H2k<sup>b</sup> promoter, hybrid intron, cDNA and SV40 polyadenylation signal. The H2k<sup>b</sup> promoter was chosen because this promoter drives constitutive expression in adult tissues and cells and appears to be particularly active in vascular endothelium.

Mice were screened for transgenesis by PCR analysis of peripheral blood and for expression of the antioxidant enzymes by reverse transcriptase PCR. The results for these experiments are shown below.

| Transgenic line                      | Number of mice | Number of transgenics | Number of expressors            |
|--------------------------------------|----------------|-----------------------|---------------------------------|
| CuZnSOD-HA                           | 76             | 10                    | 7                               |
| ECSODGPI-FLAG                        | 49             | 5                     | 5                               |
| GPx-HIS                              | 65             | 7                     | 5                               |
| CuZnSOD-HA + ECSODGPI-FLAG + GPx-HIS | 67             | 10                    | 3 triple,<br>3 double, 1 single |

Animals expressing the transgenes were mated with wild-type (WT) mice to generate offspring for further experimental analysis. Tissue lysates from WT and transgenic mice are currently being analysed for antioxidant enzyme activity as for transient transfections.

The table below shows the increase in GPx enzyme activity compared with WT mice for kidney lysates from four GPx transgenic lines.

| Transgenic line | Increase in GPx enzyme activity (%) |
|-----------------|-------------------------------------|
| TF 956-11       | 15                                  |
| TF 963-3        | 20                                  |
| TF 957-2        | 33                                  |
| TF 957-4        | 22                                  |



## Future Plans

Antioxidant enzyme activity measurements in a variety of organs from all transgenic lines will be completed. The tissue distribution of the antioxidant enzymes will also be examined using immunohistochemistry.

The functional effect of antioxidant enzyme expression on AVR will be assessed using the Gal knockout mouse heart transplantation model, which we have previously shown is an excellent small animal model of antibody-dependent rejection with many features of AVR. In this model,  $\alpha$ -1,3-galactosyltransferase knockout (Gal knockout) mice backcrossed onto a BALB/c background are injected intraperitoneally with *Leishmania* to boost the anti- $\alpha$ Gal titre. WT mice hearts transplanted into high-titre anti- $\alpha$ Gal recipients are rejected in 8–13 days and this rejection is associated with a heavy leukocyte infiltrate consisting of predominantly macrophages and NK cells.

Hearts from transgenic and WT mice will be transplanted heterotopically into high-titre anti- $\alpha$ Gal recipients and the effect of antioxidant enzyme over-expression on the function of transplanted hearts will be monitored daily by palpation. The rate and strength of contraction is graded using a scale from 0 to 4, with 4 being strong regular contraction and 0 complete cessation of function. At the time of rejection, transplanted hearts will be excised and examined histologically to determine the degree of morphological damage and the composition of the cellular infiltrate.

## Presentation

Shinkel T, Cowan P, Fiscaro N, d'Apice A. Expression of anti-oxidant enzymes as a strategy to overcome acute vascular rejection. The Transplantation Society of Australia and New Zealand Nineteenth Scientific Meeting 2001.

**Dr. Tausif Alam, Principle Investigator**

*Dr. Michael J. MacDonald, Collaborator*

*Dr. James S. Malter, Collaborator*



**University of Wisconsin, Madison, USA**

**Gene Therapy-Based Treatment for Insulin-Dependent Diabetes Mellitus**

Insulin-dependent diabetes mellitus (IDDM) is caused by selective autoimmune destruction of the insulin-producing  $\beta$ -cells of the endocrine pancreas. There are currently two therapies available: periodic injections of insulin; and transplantation of pancreas or islets. Both treatments have serious limitations. Among patients using the commonly employed insulin therapy, tight blood-sugar control has been difficult to achieve and consequently they suffer from many complications associated with long-term hyperglycemia. Pancreas or pancreatic islet transplantation can achieve precise blood glucose control, but the usefulness of these options is limited because of (a) the shortage of donor organs and (b) the side effects associated with lifelong treatment with immunosuppressive drugs. This proposal represents our continued effort towards the long-term goal of providing a replacement  $\beta$ -cell in the form of the recipient's own cells, engineered for glucose-regulated insulin secretion.

We have generated an insulin gene construct containing glucose-inducible regulatory elements (GIREs) derived from the promoter of S14, a liver protein that quickly responds to glucose, the liver-specific promoter of albumin, and the human insulin cDNA modified so that the product may be properly processed by the endogenous protease furin. This gene construct expresses insulin in hepatocytes. The insulin produced by this gene construct is biologically active, and the amount of insulin produced is time and glucose-concentration dependent. The results of preliminary studies performed using hepatocytes in cell culture and streptozotocin-treated diabetic rats were consistent with our strategy of cell engineering. The *in vivo* functional efficacy studies demonstrated glucose-regulated insulin secretion from transduced liver cells, normalized fasting

hyperglycemia and improved glucose tolerance, supporting the feasibility of gene-therapy-based treatment for IDDM. The quantity of insulin produced, however, was insufficient to completely correct hyperglycemia in diabetic rats with an unlimited access to food.

Our original proposal included modifications in the insulin gene construct and optimizations in the procedures of gene delivery to improve overall insulin production. The proposed major modifications to the insulin gene construct included addition of elements that would improve the efficiency of insulin mRNA translation, and addition of more GIREs to increase the quantity of insulin mRNA production. During the first year of support, we have been able to generate four new constructs that contain combinations of additional GIREs and translation enhancer elements. The integrity of these new constructs has been confirmed by PCR and DNA sequence analysis. Additionally, one construct containing a translation enhancer element has been successfully cloned into a replication defective adenovirus for *in vivo* testing. During this same period, the method for adenoviral *in situ* transfection of liver cells has been improved. It is now possible for us to reduce fasting blood glucose levels of treated diabetic rats to a level that is statistically indistinguishable from that of the normal rats ( $82 \pm 22$  mg/dl in treated versus  $77 \pm 9$  in normal, whereas the diabetic controls remain at  $408 \pm 52$  mg/dl).

A critical aspect of this study was validated by confirming that the insulin causing lowering of blood glucose in insulin-gene-treated diabetic rats is produced in the liver cells and is not the consequence of remaining pancreatic  $\beta$ -cell secretion due to an incomplete destruction of islet  $\beta$ -cells by streptozotocin. We have already shown by immunohistochemical methods that, under our experimental conditions, no insulin-producing cells could be detected in the pancreas of streptozotocin-treated diabetic rats. This observation, however, did not exclude the possibility of some remaining  $\beta$ -cells that were depleted of stored insulin (degranulated) due to increased demand for insulin, and therefore, could not be detected. If this were true, as part of their normal compensatory mechanism, these remaining  $\beta$ -cells under hyperglycemic challenge would overproduce insulin mRNA. We generated primers specific for rat and human insulin sequences and used them to amplify insulin cDNA from the reverse-transcribed mRNA isolated from the liver, pancreas and other control tissues of normal,

diabetic, and insulin-gene-treated diabetic rats. The results of RT-PCR analysis showed that among insulin-gene-treated diabetic rats, only the liver contained human insulin mRNA, and the pancreas lacked the rat insulin I and II transcripts. None of the other tissues tested showed the presence of human or rat insulin transcripts, confirming that the insulin-gene treatment caused liver-specific production of human insulin, and that streptozotocin completely ablated pancreatic  $\beta$ -cells. Therefore, the observed reduction in hyperglycemia was a direct consequence of human insulin produced in the liver cells of diabetic rats (manuscript in preparation).

In summary, we have made progress on all aspects of the original proposal. In the upcoming year we will evaluate the efficacy of our new constructs for insulin production in cell culture and in treating diabetic rats. We are optimistic that the newly generated insulin constructs will prove more effective in normalizing the blood glucose in diabetic rats.



## Dr. Jorge A. Bezerra, Principle Investigator

Dr. David Witte, Collaborator



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## Molecular Regulation of Liver Cell Transplantation

### Introduction

Hepatocyte transplantation is an emerging treatment modality for patients with liver failure or metabolic disease. Although the factors regulating hepatocyte transplantation are largely unknown, the plasminogen family of proteases has been proposed to regulate engraftment and clonal expansion of transplanted cells via proteolysis of the adjoining microenvironment. Therefore, we proposed a series of experiments to define the role of these proteases on the biological fate of transplanted hepatocytes.

### Results

Since our previous progress report in April 2000, we have worked to accomplish the following goals that were set for the second year of the award.

*1. Complete the studies of the role of plasminogen activators in the reparative response of the liver to acute injury.*

Since we proposed to transplant normal hepatocytes into the spleen of mice carrying the targeted inactivation of the tissue-type (tPA) and/or urokinase-type (uPA) plasminogen activator, it was essential to establish the consequences of each plasminogen activator in liver repair. To this end, we analyzed livers of tPA- and uPA-deficient mice following a single administration of an hepatotoxin. We found that tPA-deficient mice display a mild defect in hepatic repair, whereas liver of uPA-deficient mice have a more substantial delay in repair, with injury of centrilobular hepatocytes persisting up to 14 days after the toxic injury. We also observed evidence for functional cooperativity between plasminogen activators, as suggested by the increased susceptibility of mice lacking tPA and uPA simultaneously. The defective repair was not due to increased susceptibility of experimental mice to the toxin or to inadequate cellular proliferation. Instead, lack of plasminogen

activators led to the accumulation of fibrin and fibronectin within injured areas and poor removal of necrotic cells. Taken together, these data demonstrate that tPA and uPA play a critical role in hepatic repair via proteolysis of matrix elements and clearance of cellular debris from the field of injury.

### *2. Characterize the phenotype of transgenic uPA<sup>+</sup>/Plg<sup>0</sup> mice.*

In order to determine the role of uPA in the control of liver cell transplantation, we developed a line of transgenic mice that overproduce uPA and lack plasminogen (uPA<sup>+</sup>/Plg<sup>0</sup>). To completely characterize the degree of uPA overexpression in hepatocytes of uPA<sup>+</sup>/Plg<sup>0</sup> mice, we expanded the mouse colony and determined the expression of the transgene from 1 week to 1 year of age. Northern blot analysis showed that uPA is overexpressed by transgenic mice up to 7 months of age, at which time the level of expression decreases substantially. Based on these experiments, we plan to use transgenic mice younger than 7 months of age as donors of hepatocytes in transplantation experiments (see below).

### *3. Establish the plasminogen-independent role of urokinase-type plasminogen activator (uPA) in the reparative response of the liver to acute injury using uPA<sup>+</sup>/Plg<sup>0</sup> mice.*

We next determined whether overproduction of uPA compensates for loss of plasminogen *in vivo*. In these experiments, we found that development of the necroinflammatory centrilobular injury induced by administration of a hepatotoxic injury in uPA<sup>+</sup>/Plg<sup>0</sup> mice was similar in transgenic and non-transgenic mice 2 days after injury. Thereafter, livers of uPA<sup>+</sup> mice gradually restored the normal appearance, whereas the uPA<sup>+</sup>/Plg<sup>0</sup> livers continued to display the typical centrilobular injury up to 14 days after the onset of injury. Therefore, uPA overproduction does not independently induce proteolysis of the hepatic matrix and needs circulating plasminogen to direct remodelling of the liver lobule during repair.

Taken together these results demonstrate that mice lacking tPA and/or uPA are not appropriate recipients of hepatocytes following a toxic injury in view of the defective reparative response to toxins. Furthermore, transplantation experiments with hepatocytes overproducing uPA must derive from transgenic mice younger than 7 months of age because of the gradual decrease in uPA production detectable at 7 months.

## Summary and aims for the final year

We have used a series of mice carrying the targeted inactivation of genes encoding the plasminogen family of proteases to delineate their biological role in matrix remodelling during the reparative response of the liver to an acute injury *in vivo*. Our studies clearly demonstrate that this family of proteases work together to direct liver repair, with plasminogen placed at a chief regulatory position in matrix proteolysis. What remains to be explored is the ability of these proteases to augment the repopulation of diseased livers by transplanted hepatocytes.

We have initiated the following experiments to be carried out during the final year of the study.

1. Transplantation of hepatocytes overproducing uPA into non-transgenic mice before and after an acute toxic injury. These experiments will directly determine whether the overproduction of uPA by transplanted hepatocytes amplifies pericellular matrix proteolysis and facilitates incorporation of transplanted cells.
2. Transplantation of hepatocytes lacking the uPA receptor into non-transgenic mice before and after an acute injury. These experiments will define whether binding of uPA to its membrane receptor increases engraftment of transplanted hepatocytes.

## Publications

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**Prof. Alfred L. M. Bothwell, Principle Investigator**



**Yale University School of Medicine, New Haven, USA**

## **Identification of Novel Porcine Immunoregulatory Molecules**

### **Introduction**

The use in transplantation of donor organs from pigs has the capacity to solve the major problem of a lack of available donors. The long-term survival of these grafts will depend on understanding the species differences in proteins that control the recognition of lymphocytes of the recipient with the graft, especially the endothelium. In addition, the induction of proteins in response to certain cytokines may differ with respect to expression as well as the time course of induction. Some responses may have notable qualitative differences. To that end, we have begun to characterize the properties of endothelial cells that are important immunologically.

### **Aims**

The main aim of this grant is to identify novel expressed genes that are potentially involved in regulating the response of human T cells to porcine endothelial cells in a xenograft setting. The initial core effort is to identify genes in interferon- $\gamma$  or TNF- $\alpha$  treated porcine aortic endothelial cells (PAEC) that are induced. We have initiated representation difference analysis (RDA) studies with RNA from untreated cells, cells treated for 16 h with porcine interferon- $\gamma$ , and cells treated for 4 h with human TNF- $\alpha$ .

A second aim of this work is to characterize the properties of genetically altered endothelium modified to be more resistant to cytolytic cells. Isolated PAEC or human umbilical vein endothelial cells (HUVEC) that have been cast in three-dimensional collagen-fibronectin gels form an extensive network of microvessels within 24 h. When implanted into SCID mice extensive connections between murine microvessels and porcine microvessels are made resulting in perfusion of the vessels with



murine blood. Retroviral transduction of cells with Bcl-2 has resulted in PAEC that show recruitment of murine smooth muscle cells *in vivo*. Therefore, we are interested in performing the RDA analysis on the transduced PAEC versus control-vector-transduced PAEC in order to identify the important differences. Due to the availability of much more extensive human sequence data, we have first performed this analysis using HUVEC instead of PAEC.

### Progress in second year

Three independent RDA experiments have been analyzed. In the first experiment, subtractions were performed with porcine interferon- $\gamma$ -induced PAEC versus non-induced PAEC and human TNF- $\alpha$ -induced PAEC versus non-induced PAEC. The subtractions were performed using a commercial kit. In these experiments approximately 200 DNA clones were sequenced from each cytokine treatment chosen at random. In this experiment Blast searches revealed the repetitive isolation of a GTP-binding protein cDNA, which was itself a repetitive element in the porcine genome. This experiment led us to believe that the ability of the commercial kit to remove the background clones was a very significant problem. Hence, we have shifted exclusively to the use of a method originally described by Hubank and Schatz.

In the second round of subtractions we repeated the interferon- $\gamma$ -induced PAEC versus non-induced PAEC and TNF- $\alpha$ -induced PAEC versus non-induced PAEC and added Bcl-2-transduced HUVEC versus EGFP-transduced HUVEC cast in collagen gels. In this large experiment, approximately 100 clones from each subtraction were chosen at random for DNA sequence analysis. In the TNF and interferon subtractions a significant number of sequences were identified that would not have been expected with these cytokine sequences. While these clones may be interesting, we were suspicious of the quality of these subtractive procedures. We will return to this collection to re-screen it with tester and driver probes as outlined below. Analysis of the HUVEC samples revealed several interesting cDNAs that had altered expression as a consequence of Bcl-2 expression (e.g. p53).

In the third experiment we have added an important hybridization screen of the subtracted cDNA clones. The inserts from the individual clones are amplified by PCR using primers flanking the insertion site and spotted on duplicate nylon

filters. Hybridization is performed on these filters with the double-stranded DNA from the starting two RNA populations (tester TNF- $\alpha$  treated and driver untreated). The function of this screen is to eliminate cDNAs that have survived the subtractive technology but are not really unique to the tester population. In addition, hybridization of the tester probe will give some indication of the relative abundance of that individual cDNA in the total population.

We have learnt that this additional screen is extremely valuable. We have, thus far, analyzed 96 of the clones in the TNF- $\alpha$ -treated PAEC subtractions. Out of 96 clones spotted on a hybridization grid, 14 showed significant hybridization with the tester probe (TNF- $\alpha$  treated) and no hybridization with the driver probe. There are still a number of clones that did not hybridize to either probe, which might optimistically represent low-abundance RNAs that could be induced in response to TNF- $\alpha$ . This can be tested by RT-PCR and Northern blot hybridization analysis. We have sequenced 10 of the clones, and 7 of them correspond to cDNAs that are known to be induced by TNF- $\alpha$  in HUVEC. Three clones contained porcine E-selectin, and one clone each was identified for SLA class I, monocyte chemoattractant protein (MCP-1), plasminogen activator inhibitor-1 (PAI-1) and I $\kappa$ B $\alpha$ . There are three clones that show homology to human sequences. One is essentially the porcine fibronectin, which was not previously known to be induced by TNF- $\alpha$ . This information indicates that we have optimized the process for identification of induced RNAs in these cell lines.

### Summary

The analysis of the third set of subtractions indicates that we have several candidate clones for TNF- $\alpha$ -induced DNAs. Given that we currently have approximately 1000 subtracted clones, we should have over 100 that will be identified by hybridization and some new sequences should be forthcoming. The very weak hybridizing clones may be candidates as well. We expect that if the trend holds, about 70% will be already known genes but there should also be additional ones. We will focus on these clones but also perform analysis on clones from the second round of subtraction. This analysis will be extended to the porcine-interferon-treated subtracted clones and the HUVEC subtractions. Once the sequence analysis is completed the clones will be assessed for induction by RT-PCR and Northern blotting.

## **Dr. Julie Dechanet, Principle Investigator**

*Dr. Jean-Francois Moreau, Co-Applicant*

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## **Study of the Role of Gamma-Delta T Lymphocytes in the Immune Response Directed against Human Cytomegalovirus**

### **Introduction**

Numerous studies analysed the immune response against cytomegalovirus (CMV) either in humans or in mice and pointed out the major role played by NK cells and specific anti-viral CD8<sup>+</sup> αβ T cells. Before our work, no attention was paid to γδ T cells though they have been implicated in numerous anti-infectious immune responses in which they sometimes play a protective role for the host. The ligands recognised by γδ TCR have been clearly identified for Vγ9/Vδ2 T cells (the main γδ T cell population in the peripheral blood) as non-peptidic phosphorylated antigens expressed by many procaryotic and eucaryotic cells. The remaining γδ T cells (expressing any of the other 7 Vδ with any of 6 Vγ chains and referred to as Vδ2<sup>neg</sup> in this report) are essentially tissue localised in spleen and mucosal epithelia, whereas their ligands are far less known. From 1997 on, we observed a dramatic and persistent expansion of γδ T lymphocytes in the peripheral blood of renal transplanted patients when they develop a CMV infection. Only Vδ2<sup>neg</sup> cells are affected by this amplification (mainly Vδ1, which is the major Vδ2<sup>neg</sup> population in the body, but also Vδ3 and Vδ5 cells). Some patients display among their peripheral CD3<sup>+</sup> T cells up to 50% Vδ2<sup>neg</sup> γδ T lymphocytes whereas these cells do not exceed 1% in healthy individuals. These values persist for years after infection and never return to basal levels. The Vδ2<sup>neg</sup> T cell repertoire is highly restricted (monoclonal in some patients), suggesting an antigen-driven selection of these cells during CMV infection. CMV-infected cell lysates induce γδ T cell proliferation among PBMC *in vitro*. These results indicate that Vδ2<sup>neg</sup> γδ T cells may recognise an antigen induced during cellular infection by CMV. The aim of our project is to understand the role played by Vδ2<sup>neg</sup> γδ T cells in the anti-viral immune response, notably through the identification of the antigens recognised by these cells.

## Results

Expansion of V $\delta$ 2<sup>neg</sup>  $\gamma\delta$  T cells was also found in other immunosuppressed patients: hepatic, pulmonary, bowel or bone marrow transplanted patients. V $\delta$ 2<sup>neg</sup>  $\gamma\delta$  T cells express the same HLA as the recipient of kidney, lung or intestine grafts, demonstrating that they do not originate from these organs. This was a surprising result as V $\delta$ 2<sup>neg</sup>  $\gamma\delta$  T cells are mainly localised in mucosal epithelia, which are also the preferential sites for CMV replication. They may come from the spleen where V $\delta$ 2<sup>neg</sup>  $\gamma\delta$  T cells represent about 30% of T cells. The percentage of V $\delta$ 2<sup>neg</sup>  $\gamma\delta$  T cells is also slightly but significantly enhanced in the blood of healthy CMV seropositive persons compared with seronegative ones, suggesting their anti-viral implication also in immunocompetent individuals. Comparison of the V $\delta$ 1 and V $\delta$ 3 T cell repertoires between seropositive and seronegative healthy donors is under investigation.

We carried out a prospective study on 64 CMV<sup>+</sup> patients to determine the relationships between CMV-infection evolution and the kinetics of  $\gamma\delta$  T-cell expansion. Patients with late  $\gamma\delta$  T-cell expansion (>45 days after CMV infection) had significantly longer ( $p<0.0001$ ) and higher pp65 antigenemia ( $p<0.0003$ ), and more symptomatic CMV disease than patients with early expansion. Furthermore,  $\gamma\delta$  T-cell expansion is concomitant with the resolution of CMV infection and disease, regardless of the CMV serological status of donor and recipient before transplantation. These observations point to the percentage of  $\gamma\delta$  T cells as a reliable prognosis factor to predict the resolution of CMV infection. Moreover, these results strongly suggest that  $\gamma\delta$  T cells play a protective role against CMV. Since last year, similar results were obtained in a study on 35 new patients receiving prophylactic oral anti-viral treatment.

During the two last years, we generated a large panel of V $\delta$ 2<sup>neg</sup>  $\gamma\delta$  T cell lines and clones isolated from CMV-infected transplanted patients. All of these clones were cytotoxic in redirected killing experiments performed with an anti-CD3 mAb. Difficulties in optimising culture conditions for these very low proliferative potential cells and in finding the appropriate target cell line for CMV infection delayed project progress.

Nevertheless, during the second year we demonstrated that some of these T cell lines and clones specifically react against U373 cells (human astrocytoma cell line) only when it is infected with CMV. In this instance, they are able to produce TNF- $\alpha$ , IFN- $\gamma$  and GM-CSF. The optimal reactivity of clones is obtained 72 h after infection, and it is inhibited by the presence of an anti-TCR mAb. Cytotoxicity of V $\delta$ 2<sup>neg</sup>  $\gamma\delta$

T cells against CMV-infected U373 cells is also enhanced compared with non-infected cells. Reactive T cells reached 20% among some of the polyclonally generated V $\delta$ 2<sup>neg</sup>  $\gamma\delta$  T cell lines, suggesting a high frequency of these cells *in vivo*.

We undertook the generation of V $\delta$ 2<sup>neg</sup>  $\gamma\delta$  TCR transfected Jurkat cell lines that is necessary (1) to avoid technical limitations of poorly proliferating V $\delta$ 2<sup>neg</sup>  $\gamma\delta$  T cells, (2) to prove the involvement of the TCR in anti-CMV  $\gamma\delta$  T cell activation, and (3) to screen blocking anti-ligand mAbs (see below). We chose the J.RT3-T3.5 Jurkat cell line, which does not express original  $\alpha\beta$  TCR nor CD3 on its surface, and pREP plasmids (as used used by Brenner et al.) to transfect whole  $\gamma$  and  $\delta$  TCR chains containing V $\gamma$ 9 and V $\delta$ 2 variable domains, respectively. V $\delta$  and V $\gamma$  cDNA (isolated from three V $\delta$ 2<sup>neg</sup>  $\gamma\delta$  T cell clones from two CMV<sup>+</sup> transplanted patients and one CMV<sup>-</sup> control patient) have already been cloned in pREP expression vectors and transfection/selection steps are in progress.

We also initiated the construction of soluble tetrameric V $\delta$ 2<sup>neg</sup>  $\gamma\delta$  TCR (single-chain Fv construction) that will be used (1) to easily detect the presence of the ligand on cell surface or in cell lysates, (2) to block TCR/ligand interaction for detailed analyses of the anti-CMV reactivity of V $\delta$ 2<sup>neg</sup>  $\gamma\delta$  T cell clones, and (3) for structural studies of V $\delta$ 2<sup>neg</sup>  $\gamma\delta$  TCR. HEK cells have been transfected with pEDr plasmids containing cDNA coding for variable regions of  $\delta$  and  $\gamma$  chains from an anti-CMV V $\delta$ 2<sup>neg</sup>  $\gamma\delta$  T cell clone. Characterisation of the recombinant soluble TCR is underway.

### Aims for the final year

For the final year our attention will be focused on the identification of the antigenic ligands recognised by the TCR of V $\delta$ 2<sup>neg</sup>  $\gamma\delta$  T cell clones. Detailed analysis (specificity of CMV, use of deleted CMV strains, test of stressed target cells, role of NK receptor molecules, etc.) of the *in vitro* anti-CMV reactivity of V $\delta$ 2<sup>neg</sup>  $\gamma\delta$  T cell clones will be accomplished in order to publish this work before the end of the year. We then aim to obtain the V $\delta$ 2<sup>neg</sup>  $\gamma\delta$  TCR transfected cell lines necessary for the screening of mAbs that will be generated against CMV-infected U373 cells. mAbs will be selected that are able to block the reactivity of Jurkat cell lines against CMV-infected U373 cells as they should be directed against the ligand. These mAbs could be used to immunoprecipitate the ligand from CMV-infected U373 cells and thus will be of great help for molecular weight determination, for microsequencing of peptides if it is a protein, or for chromatography purification. Concurrently, we plan to complete the construction of the tetrameric soluble V $\delta$ 2  $\gamma\delta$  TCR.

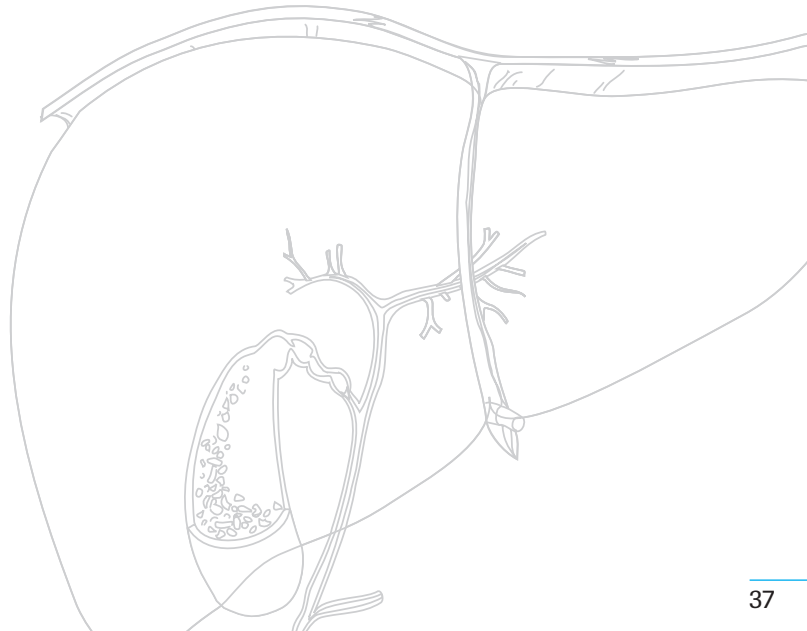
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Dr. Calvin Harley, Dr. Walter Funk, Dr. Nam Kim,  
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## The Role of Senescence and Telomere Shortening in Chronic Rejection

### Aim

To examine cell aging ('senescence') mechanisms in transplantation. Our hypothesis has been that kidneys age by mechanisms similar to 'replicative senescence' in culture, and the stresses of transplantation aggravate these changes.

### Results

The focus of our work during the second year of support has been on the importance of genes responsible for regulating senescence in human and rodent kidneys. The cyclin-dependent kinase (CDK) inhibitor p16<sup>INK4a</sup> is a mediator of cellular senescence *in vitro*. It plays a key role at the G1 cell-cycle checkpoint by inhibition of CDK4 and CDK6, thereby blocking cell proliferation. Measurement of p16<sup>INK4a</sup> mRNA expression was done using quantitative RT-PCR. Gene expression was measured in 51 human kidney samples from individuals of various ages (normal histology:  $n=37$ ; abnormal histology:  $n=14$ ). In addition, specimens (kidney, spleen, brain and heart) from 32 rats of three different age groups (1, 9, 24 months) were investigated. p16<sup>INK4a</sup> expression was high in kidneys from older humans. Regression analysis excluding samples with histologic abnormalities revealed a significant increase for cortex ( $p<0.05$  for 18S RNA,  $p<0.001$  for HPRT normalized values), but not for medulla ( $p=0.82$  for 18S RNA,  $p=0.38$  for HPRT normalized values). However, kidneys with abnormal features tended to show high p16<sup>INK4a</sup> levels even in younger individuals.

In rat kidneys, the mean values ( $\pm$ SD) for p16<sup>INK4a</sup>/18SRNA were  $1.59 \times 10^{-10}$  ( $\pm 5.29 \times 10^{-10}$ ) for 1-month-old,  $4.38 \times 10^{-9}$  ( $\pm 2.92 \times 10^{-9}$ ) for 9-month-old, and  $3.16 \times 10^{-7}$  ( $\pm 8.85 \times 10^{-7}$ ) for 24-month-old rats. Only one of 11 rat kidney samples in the

1-month group showed p16<sup>INK4a</sup> expression, but very low. Nine-month-old rats had detectable levels in all 10 animals. Highest levels were found in 24-month-old rats. The latter group showed quite high variability in p16<sup>INK4a</sup> levels. The difference between the three age groups was highly significant ( $p < 0.001$ ). Similar significant increases were seen for the other three tissues investigated (spleen  $p < 0.001$ ; brain  $p < 0.05$ ; heart  $p < 0.05$ ). Both spleen and heart showed p16<sup>INK4a</sup> expression even in the 1-month-old group, whereas for brains from 1-month-old rats were p16<sup>INK4a</sup> mRNA negative. These data support the hypothesis that p16<sup>INK4a</sup> is not normally expressed in young kidney, but is induced with aging and disease, compatible with its putative role in cellular senescence.

We assessed the importance of a known *in vivo* marker for senescence: senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -GAL). SA- $\beta$ -GAL staining was found to different degrees in all human specimens investigated. Although very intense SA- $\beta$ -GAL staining seemed to be more common in specimens from older individuals, statistical analysis revealed no difference between specimens from young and old individuals. SA- $\beta$ -GAL staining for rat specimens of different ages was statistically significant ( $p < 0.005$ ). Mean staining densities were 0.003 ( $\pm 0.002$ ) for 1-month-old, 0.008 ( $\pm 0.003$ ) for 9-month-old, and 0.02 ( $\pm 0.007$ ) for 24-month-old rats.

We have shown previously that telomere attrition occurs in human kidney cortex, compatible with a role for telomere shortening and replicative senescence in aging in human renal cortex. We established the use of pulse field gel electrophoresis (PFGE) for investigation of telomere restriction fragments (TRF) with an average length of 40 kb in rodents. Using this technique we investigated kidney samples derived from normal rats at different ages. The mean  $\pm$  SD TRF length was  $38.5 \pm 1.0$  for 1-month-old,  $40.6 \pm 4.8$  for 9-month-old, and  $35.1 \pm 6.0$  for 24-month-old rat kidneys (not significant).

In summary, features associated with cellular senescence *in vitro* develop with age in rat and human kidneys. Thus cellular senescence may contribute to the renal aging phenotype, including end-stage diseases, cancer, and poor performance following transplantation. As in senescent cells *in vitro*, kidney senescence *in vivo* shows species differences: human kidneys manifest telomere shortening whereas rodents do not, suggesting that telomere shortening contributes to human but not rat senescence. Thus senescence in human and rodent kidney displays both similarities and fundamental differences. This has to be taken into account when



analyzing injury and transplantation models in rodents. The correlation between the species differences *in vitro* and *in vivo* supports the concept that cellular (replicative) senescence of key cells contributes to the renal aging phenotype, and thus may contribute to the problem of excessive loss of function in older kidneys when they are subjected to transplantation or other stresses.

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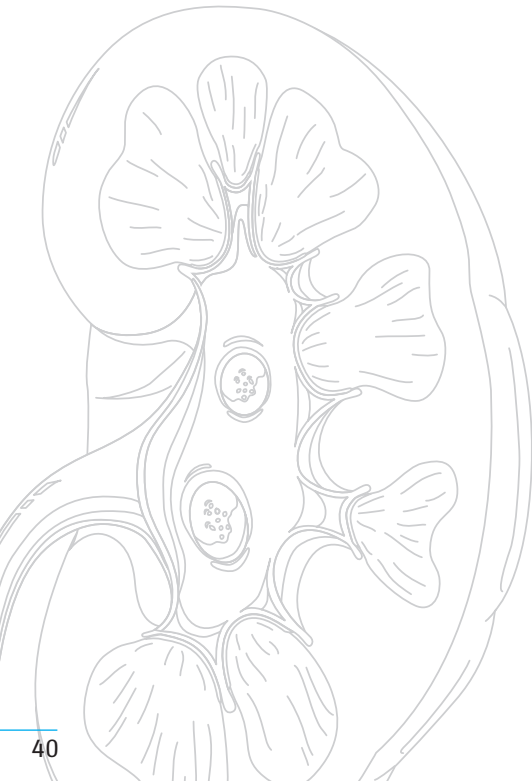
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**Identification of a Novel Protein Involved  
in Rejection of Transplanted Organs**

The principal goal of our application is to identify a role for two key transcription factors (STAT5a/b) involved in mediating T-cell activity and allograft rejection. Within this proposal we suggest three approaches to inactivate these proteins and ultimately determine their effects *in vitro* and *in vivo* using T-cell cultures and rat heart allograft transplant models, respectively. At the conclusion of the first year of support, we have made significant strides towards attaining the first major aim of the proposal.

**Specific Aim 1. Selective inhibition of Jak3 blocks STAT5a/b  
activation and T lymphocyte function**

We have satisfied nearly all of the proposed sub-aims within Specific Aim 1 by identifying two selective inhibitors (AG-490 and PNU156804) that block STAT5a/b tyrosine phosphorylation and consequent activation. Both agents disrupted STAT5a/b activity following stimulation of T cells with various T-cell growth factors, including interleukin (IL)-2 and other cytokine family members (IL-7, IL-9 and IL-15). We showed that AG-490 and PNU156804 disrupt T-cell proliferation and Janus-kinase-mediated activation of STAT5a/b. Moreover, we found that STAT5a/b are unable to translocate to the nucleus and bind DNA, thus ablating their ability to regulate T-cell-mediated gene transcription. Using Lewis to ACI rat heart transplant models we demonstrated *in vivo* that STAT5a/b DNA-binding activity is disrupted in lymphocytes isolated from treated recipients. Additional evidence was also presented to support our model for a role for STAT5a/b in later T-cell signaling events (signal 3). We draw this conclusion because T cell activation was not blocked following inhibition of STAT5a/b, as measured by unaltered mRNA levels

of the  $\alpha$ -chain of the IL-2 receptor, which is only expressed on activated T cells. We also showed that inhibition of this effector pathway *in vivo* significantly prolongs allograft survival alone, but acts synergistically when used in combination with sub-therapeutic doses of cyclosporin A (CsA), a signal 1 inhibitor. Moreover, we established that inhibition of STAT5a/b with AG-490 or PNU156804 plus rapamycin (a signal 3 inhibitor) is not synergistic, but additive. We also demonstrated that inhibition of the Janus kinase 3/STAT5a/b pathway reduces mononuclear cell infiltration and damage of heart allografts in the transplanted recipients.

### **Specific Aim 2 (Year 2). Measure immunosuppressive potential of dominant negative forms of STAT5a/b**

As a second approach to disrupting STAT5a/b activity, we are generating dominant-negative constructs of STAT5a/b to inhibit T cell activity. Ongoing studies are being performed within the laboratory to attain this goal. At present we are expanding our adenoviral vectors to include retroviral packaging systems. Low transfection efficiency of the former necessitated the optimization of this system.

### **Specific Aim 3 (Year 3). Selective inhibition of STAT5a/b by antisense oligonucleotides blocks T cell function and rejection of transplanted organs**

No work has been published on the use or design of antisense oligonucleotides for STAT5 protein suppression. We have accomplished the first major step in the design and synthesis of STAT5 antisense oligonucleotides using standard gene walk methodology. Studies have been set in motion to screen through these putative antisense oligonucleotides for ones that optimally suppress STAT5a, STAT5b and/or STAT5a/b protein expression in lymphocytes.

We expect to prove that selective inhibition of STAT5 by antisense oligonucleotides will inhibit STAT5 mRNA/protein expression, T-cell function in response to IL-2 and other cytokines, and allograft rejection. Since previous studies by our group and others have shown that phosphorothioate-methoxyethyl-modified oligonucleotides can be delivered effectively *in vitro* and *in vivo*, we expect this approach will prolong allograft survival with greater specificity and less toxicity.

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### **Effect of T Cell Costimulatory Blockade and Bone Marrow Transplantation on Organ Transplant Rejection**

Substantial experimental and clinical evidence demonstrates that intestinal allografts elicit an unusually strong recipient immune response. Consequently, immunosuppressive strategies that inhibit rejection or even promote long-term allograft acceptance in some transplant models fail to alter the rate or severity of intestinal allograft rejection. We have previously shown that although blockade of the CD28/B7 and/or CD154/CD40 costimulatory pathways promotes long-term survival of cardiac allografts in mice, these agents do not inhibit the rejection of intestinal allografts in the same strain combination. We have shown this to be due to the relative resistance of CD8<sup>+</sup> T cells to blockade of costimulatory pathways.

One approach that has been used to induce the long-term acceptance of transplanted organs is to combine organ transplantation with donor bone marrow transplantation. In the past the clinical application of this strategy has been limited by the toxicities of the conditioning regimens required to facilitate bone marrow engraftment. The initial aims of this project were to determine whether combined intestinal and bone marrow transplantation could inhibit the rejection of allogeneic intestinal grafts and to identify less toxic and therefore potentially more clinically applicable approaches to promoting engraftment of donor bone marrow cells.

In collaboration with Drs. Christian Larsen and Thomas Pearson (Emory University, Atlanta, GA, USA) we have investigated the use of busulfan as a conditioning agent to allow engraftment of donor bone marrow cells. Busulfan is an alkylating agent that causes preferential depletion of early hematopoietic stem cells and is used clinically as part of a conditioning regimen for allogeneic bone marrow transplantation. In these experiments it is used in place of other more toxic conditioning strategies, such as TBI. The current protocol involves the transplantation of  $20 \times 10^6$  bone marrow cells on day 0 together with combined blockade of

the CD28/B7 and CD154/CD40 costimulatory pathways using CTLA4-Ig and MR1 administered on days 0, 2, 4 and 6. Recipient mice receive busulfan on day 5 and repeat bone marrow infusion on day 6 ( $20 \times 10^6$  bone marrow cells). Our data suggest that intestinal transplantation can be performed at the initiation of therapy (day 0) as well as at later time points (e.g. day 20). Combined costimulatory blockade, busulfan, and bone marrow transplantation completely abrogated rejection in allografts examined at day 50. In contrast, combined costimulatory blockade alone had no effect on intestinal allograft rejection with all wild-type recipients developing moderate to severe rejection by day 14.

The regimen of costimulatory blockade, bone marrow transplantation, and busulfan resulted in multi-lineage hematopoietic macrochimerism, including granulocytes, macrophages, dendritic cells, T cells, B cells, and NK cells. T cells from treated mice displayed donor-specific unresponsiveness *in vitro* in mixed lymphocyte reactions but maintained normal responses to third party alloantigens. Making use of differences in donor and recipient expression of I-E molecules our data demonstrate that the prolongation of allograft acceptance in this model was associated with substantial deletion of donor-reactive T cells. Superantigens encoded by MMTV 8 and 9 bind I-E molecules and delete CD4<sup>+</sup> T cells expressing V $\beta$ 5 and V $\beta$ 11. Thus I-E<sup>+</sup> strains delete V $\beta$ 5 and V $\beta$ 11 CD4<sup>+</sup> cells while I-E<sup>-</sup> strains do not. Survival of I-E<sup>+</sup> intestinal allografts and bone marrow cells in I-E<sup>-</sup> recipients was associated with significant depletion of V $\beta$ 5 and V $\beta$ 11 CD4<sup>+</sup> T cells that increased over time.

These data demonstrate that combining costimulatory blockade with donor bone marrow infusion and busulfan promotes development of stable multi-lineage hematopoietic macrochimerism that is associated with significantly prolonged survival of highly immunogenic intestinal allografts. Based on the *in vitro* analysis of recipient T cells this effect appears to be donor specific. Although the responsible mechanism(s) has yet to be fully elucidated, our data suggest that deletion of donor-reactive T cells is at least partially responsible. In addition to the obvious advantages of avoiding long-term treatment with relatively non-specific immunosuppressive drugs, this approach also offers other advantages when compared with more classical methods of inducing macrochimerism. First, busulfan has relatively minor effects on mature hematopoietic cells. As used in this protocol, busulfan results in a mild, transient leukopenia and thrombocytopenia (Andrew Adams,

laboratory of CPL and TCP). Secondly, relatively few bone marrow cells are required. Thirdly, this approach should be directly applicable to clinical organ transplantation using organs from cadaveric donors, since no treatment is required prior to the time of transplantation.

The aims for the second year of the study are to determine the longevity of this effect, confirm the donor specificity of this effect using more rigorous *in vivo* methods, and investigate in more detail the mechanisms responsible for this effect. While deletion of donor-reactive T cells occurs and could logically be predicted to be at least partially responsible for the inhibition of rejection in this model, data from other groups suggest that regulatory cells and/or peripheral tolerance may also be involved. Finally, given the failure of many strategies to directly inhibit CD8<sup>+</sup> T-cell-mediated rejection, we will directly examine the effects of this approach on isolated CD8<sup>+</sup> and CD4<sup>+</sup> T cells.



**Dr. Jon S. Odorico, Principle Investigator**

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### **Derivation of Insulin-Producing Cells from Embryonic Stem Cell Lines**

Embryonic stem (ES) cells are pluripotent cell lines derived from the inner cell mass of pre-implantation blastocyst-stage embryos. They can be genetically manipulated with relative ease by routine procedures and can be expanded many fold in culture. Another property of ES cell lines is their ability to differentiate into a variety of embryonic cell types *in vitro*, including neurons, glia, epithelia, adipocytes, chondrocytes, endothelial cells, hematopoietic cells, melanocytes, many muscle lineages, yolk sac and pancreatic islet cells. This property of ES cells is recognized as a valuable tool for studying many facets of mammalian development *in vitro*. The overall goal of this project is to develop an *in vitro* ES-cell-based differentiation system to study pancreatic islet development. In addition, an ES-cell-based strategy could permit the generation of an unlimited supply of insulin-producing  $\beta$  cells from an abundant, renewable and readily accessible source for transplantation.

We and others have shown that mammalian ES cells are capable of activating the expression of pancreatic islet- and endoderm-specific genes and can differentiate into insulin-secreting  $\beta$  cells<sup>1-4</sup>. However, under standard culture conditions, islet cells develop infrequently among a mixture of differentiated cell types. Therefore, it would be of interest to devise methods to enrich or purify islet cells from ES cells. The specific aims of this project are to enhance the pancreatic endocrine differentiation of ES cells using a combination of genetic and biochemical approaches, and to derive homogeneous populations of pancreatic islet cells.

### **Results**

Our initial work has been aimed at determining whether ES cells are capable of differentiating into all islet endocrine cell lineages, establishing the timing of appearance of endocrine cells, and identifying whether cells phenotypically resembling



pancreatic islet precursor cells exist in differentiating ES cell cultures. Furthermore, based on genetic selection strategies developed for selecting specific cell types from heterogeneous ES cell cultures, we have designed a double-selection transgene construct that will facilitate the selection and enrichment of pancreatic precursor cells expressing Pdx1. These results indicate the capacity of ES cells to differentiate into islet endocrine cells and establish the feasibility of identifying, isolating and expanding islet precursor cells from ES cells.

### **ES cells exhibit pancreatic islet lineage differentiation *in vitro***

In order to study the ability of ES cells to differentiate into pancreatic islet lineages, we examined *in vitro* cultures of spontaneously differentiating mouse ES cells at various times after plating embryoid bodies (EB) for evidence of pancreatic islet differentiation using RT-PCR analysis and confocal immunofluorescence microscopy.

Murine D3 ES cells were grown and allowed to differentiate in suspension culture into EB for 7 days in media containing 15% fetal calf serum without added growth factors. Under these conditions, initial commitment to early germ layer lineages occurs and genes characteristic of ectoderm, mesoderm and endoderm are expressed. Further differentiation occurs when EBs are directly plated to adhesive tissue culture plastic without dispersion.

These studies showed that there is a time-dependent induction of pancreatic islet endocrine hormones. Immunofluorescence studies demonstrated that the absolute number of cells expressing islet amyloid polypeptide (IAPP) increased progressively over time, first appearing in significant numbers 17 days after EB differentiation.

Somatostatin (SOM)<sup>+</sup> cells also first appeared between 14 and 17 days following EB differentiation. In contrast, glucagon (GLUC)<sup>+</sup> cells developed earlier, with a significant population of cells appearing by day 14. After 28 days the frequency of GLUC<sup>+</sup> and SOM<sup>+</sup> cells decreased, whereas the frequency of IAPP<sup>+</sup> cells was still increasing. The early appearance of GLUC<sup>+</sup> cells among differentiated ES cell derivatives is consistent with the *in vivo* observation of GLUC<sup>+</sup> cells arising in the early pancreatic bud epithelium. These preliminary studies indicate that the key time interval to study in more detail for the appearance of islet precursor cells is between 10 and 17 days. Moreover, they quantify the time-dependent differentiation of islet endocrine cells from ES cells.

Endocrine cells in ES cell cultures, particularly IAPP<sup>+</sup> cells, were generally clustered in discrete regions rather than diffusely distributed throughout the culture dish. At least two possibilities could account for this finding: ongoing specific local inductive processes from a precursor cell; or proliferation of IAPP<sup>+</sup> cells. Based on these preliminary studies we cannot distinguish these two possibilities. Future studies will test the hypotheses that IAPP<sup>+</sup> cells undergo progressive differentiation from a precursor cell pool.

Each of the four major islet hormones are produced by individual ES cell derivatives. Interestingly, cells co-expressing insulin (INS) and GLUC during ES cell differentiation in vitro were not detected. Though SOM<sup>+</sup> and IAPP<sup>+</sup> cells were also present, we did not observe any cells co-expressing any combination of the four major islet hormones. Moreover, Pdx1<sup>+</sup> pancreatic precursor cells and early peptide YY<sup>+</sup> islet endocrine cells are present in ES cell cultures. Because all four major islet endocrine cell types differentiate simultaneously and in close proximity, ES cell cultures provide a useful in vitro model of islet cytodifferentiation.

### **ES cell derivatives express islet transcription factor genes**

Pdx1, Ngn3 and Nkx6.1 are critical transcription factors involved in pancreatic islet development. To determine whether ES cell progeny expressed these and other islet-restricted transcription factor genes, we performed RT-PCR analysis. Pdx1 is expressed at low levels during early phases of ES cell differentiation, and following establishment of endoderm differentiation is upregulated. Many other islet cell transcription factors genes, such as Ngn3, Nkx6.1, Nkx2.2, Pax4, Pax6, Isl1 and NeuroD, are also expressed in differentiated ES cell progeny. In contrast, expression of p48-PTF1, a transcription factor required for exocrine pancreas development, was not detected under these conditions.

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**Role of Viral Chemokine Receptors in  
Cytomegalovirus-Accelerated Transplant Vascular Sclerosis**

Transplant vascular sclerosis (TVS), the vascular lesion associated with chronic graft rejection, is characterized by concentric neointimal smooth muscle cell (SMC) accumulation resulting in vessel narrowing and ultimately graft failure. Cytomegalovirus (CMV) accelerates the development of TVS in human and rodent solid organ transplant recipients, and is associated with higher rates of graft rejection, and earlier onset and greater severity of TVS. The mechanism behind CMV's link to TVS may involve recruitment of inflammatory cells and SMC by chemokines.

Chemokines are a group of inducible, low-molecular-weight cytokines that promote cellular migration and activation. These molecules and their receptors play a major role in the development of vascular disease, as they are important stimuli for the migration and proliferation of SMC. The expression of CC-chemokines, which include MCP-1, MIP-1a and b, and RANTES, are upregulated in the vascularized graft during ischemia/reperfusion injury, acute rejection, and chronic rejection. In contrast, long-term graft acceptance has been attributed to the absence of chemokine expression, thus substantiating a major role for chemokines in allogeneic graft rejection and during the development of TVS. Importantly, CMV encodes a CC-chemokine and CC-chemokine receptors and modulates host chemokine expression *in vivo*.

**Aims**

This project aims to determine the role of CMV in the acceleration of TVS formation in a rat heart transplantation model by studying the kinetics of TVS, virus presence and CC-chemokine expression in the grafts. In addition, the contribution of the rat CMV (RCMV) CC-chemokine receptor R33 to TVS will be studied. Lastly, *in vitro* studies will be performed to determine the role of R33 in RCMV-induced SMC migration.

## Results

To elucidate the mechanisms involved in CMV-accelerated TVS, we first determined the kinetics of TVS in graft hearts with or without RCMV infection. For these studies, F344 hearts were transplanted heterotopically into Lewis recipients, treated with low-dose cyclosporin A (CsA), and acutely infected with RCMV. Syngeneic transplant recipients and uninfected allograft recipients served as controls. Animal grafts and native hearts were harvested at days 7, 14, 21, 35 and 45 days (45 days is the mean time to develop chronic rejection and TVS in CMV-infected allograft recipients). The transplanted hearts were evaluated histologically, and TVS was assessed morphologically as the mean percentage of vessel occlusion (neointimal index, NI). Graft vessels showed endothelialitis in the RCMV-infected, but not in the uninfected, allogeneic recipients at 7 and 14 days post-op. TVS was detected at 21 days post-op with little difference between infected and uninfected allogeneic recipients (NI=38 vs NI=35). However, at days 35 and 45 post-op, RCMV-infected recipient heart graft vessels showed a dramatic increase in the severity of TVS (NI=64 and 82) compared with uninfected allografts (NI=30 and 43,  $p < 0.001$ ). These data suggest that the effect of RCMV on acceleration of TVS is manifested between 21 and 35 days post-op.

Next, graft and native hearts were analyzed for the presence of viral DNA using nested PCR techniques and quantitative PCR. RCMV DNA was found in both the graft and native hearts at all times tested, whereas viral DNA was only detected in the blood until 14 days post-op.

In order to investigate what effect RCMV infection has on chemokine expression kinetics, we determined chemokine expression in grafts +/- RCMV infection using RT-PCR and quantitative PCR techniques. Chemokine profiles in graft hearts differ dramatically with or without RCMV infection. In the infected animals, graft hearts showed high expression of MCP-1 and MIP-1 $\alpha$  and  $\beta$  at all times except at the time of graft failure due to chronic rejection (day 45). However, high MCP-1 and MIP-1 $\alpha$  and  $\beta$  expression were delayed in graft hearts from uninfected animals, and chemokine expression was lower in their native hearts. In infected grafts, RANTES expression was upregulated early (day 7) and was prolonged throughout the process of chronic rejection.

These data suggest that early events (endothelialitis and upregulation of CC-chemokines, day 7) and higher chemokine expression throughout the development of TVS lead to RCMV acceleration of the disease process. RCMV is present within the graft tissues during all stages of RCMV-accelerated TVS. In the

RCMV-infected graft recipients, chemokine expression was accelerated and increased over uninfected recipients, suggesting that chemokines play an important role in this process. The early increase in, and in some cases (e.g. RANTES) prolonged, expression of chemokines, during the chronic rejection process in RCMV-infected recipients accelerates graft rejection by enhanced heart graft recruitment of inflammatory and SMC.

The aims of the second year of this project will be to determine the specific cell types present in the graft vascular lesions, and which of these harbor the virus, at various time points in the evolution of chronic rejection by *in situ* and immunohistochemical techniques. In addition, using a panel of antibodies to different kinetic classes of RCMV, viral gene expression will be mapped during chronic rejection.

### Presentations and Publications

De La Melena VT, Cook JW, Yin Q, Kreklywich CN, Streblov DN, Nelson JA, Orloff SL. The kinetics and development of CMV-accelerated transplant vascular sclerosis in rat cardiac allografts is linked to early presence of virus in the graft. *Transplantation* 2000; 69(8): S347.

De La Melena VT, Cook JW, Yin Q, Kreklywich CN, Streblov DN, Nelson JA, Orloff SL. The role of chemokines in the development of CMV-accelerated transplant vascular sclerosis. Presented at the 34th Annual Meeting for the Association of Academic Surgeons, Tampa, Florida, November 2-4, 2000.

De La Melena VT, Cook JW, Yin Q, Kreklywich CN, Streblov DN, Nelson JA, Orloff SL. The role of chemokines in the development of CMV-accelerated transplant vascular sclerosis. Presented at the 87th Annual Meeting of The North Pacific Surgical Association, Coeur D'Alene, Idaho, November 10-11, 2000.

De La Melena VT, Streblov DN, Kreklywich CN, Soderberg-Naucler C, Yin Q, Bruggeman CA, Cook JW, Nelson JA, Orloff SL. Up-regulation of CC-chemokines parallels the development of CMV-accelerated transplant vascular sclerosis. To be presented at the 21st Annual Meeting of the Surgical Infection Society, Snowbird, Utah, May 3-5, 2001.

De La Melena VT, Kreklywich CN, Streblov DN, Yin Q, Cook JW, Nelson JA, Orloff SL. The kinetics and development of CMV-accelerated transplant vascular sclerosis in rat cardiac allografts is linked to early presence of virus in the graft. *Surg Forum* 2000; LI: 350-352.

De La Melena VT, Kreklywich CN, Streblov DN, Yin Q, Cook JW, Soderberg-Naucler C, Bruggeman CA, Nelson JA, Orloff SL. The kinetics and development of CMV-accelerated transplant vascular sclerosis in rat cardiac allografts is linked to early increase in chemokine expression and presence of virus. *Transpl Proc* 2001; 33: 1822-1823.

Kreklywich CN, Streblov DN, De La Melena VT, Yin Q, Bruggeman CA, Vink C, Soderberg-Naucler C, Nelson JA, Orloff SL. Rat cytomegalovirus infection alters chemokine expression and accelerates transplant vascular sclerosis. Presented at the Keystone Symposia Chemokine and Chemokine Receptors, Taos, New Mexico, February 16-21, 2001.

Streblov DN, De La Melena VT, Kreklywich C, Yin Q, Bruggeman CA, Vink C, Soderberg-Naucler C, Nelson JA, Orloff SL. The kinetics and development of CMV-accelerated TVS in rat cardiac allografts is linked to early presence of virus in the graft. Presented at The International Herpes Virus Workshop, Portland, OR, July 31-August 4, 2000.

**Dr. Miguel P. Soares, Principle Investigator**

*Prof. Fritz Bach, Co-Applicant*



**Harvard Medical School, Boston, USA**

### **Heme Oxygenase-1:**

#### **An Anti-Inflammatory Molecule that Promotes Organ Graft Survival**

Mouse-to-rat cardiac transplants survive long term under brief inhibition of complement activation by cobra venom factor (CVF) and continuous T cell immunosuppression by cyclosporin A (CsA) (Koyamada et al., 1998), a phenomenon we refer to as accommodation (Soares et al., 1999). We have shown that expression of the protective gene heme oxygenase-1 (HO-1) in these grafts is essential to ensure accommodation (Soares et al., 1999). We have also shown that the protective effect of HO-1, in terms of sustaining graft survival, relies on its ability to catabolyze heme into the gas carbon monoxide (CO) (Sato et al., 2001).

In the absence of HO-1 expression (Soares et al., 1998) or under inhibition of HO-1 enzymatic activity by tin protoporphyrin (SnPPIX) (Sato et al., 2001) mouse hearts transplanted into rats treated with CVF plus CsA are rejected through a process that is characterized by widespread endothelial cell (EC) apoptosis. CO suppresses graft rejection by a mechanism that remains to be fully elucidated but is associated with the inhibition of EC apoptosis. This set of observations suggests that the protective effect of CO may be mediated through a process that involves the inhibition of EC apoptosis.

In the past year we have investigated the molecular mechanism underlying the protective effect of CO in terms of suppressing EC apoptosis. We found that over-expression of HO-1 or induction of endogenous HO-1 expression by heme protects cultured EC from apoptosis mediated through TNF- $\alpha$ , etoposide or serum starvation (Brouard et al., 2000). When HO-1 enzymatic activity is blocked by SnPPIX or the action of CO is inhibited by hemoglobin, HO-1 no longer prevents EC apoptosis, while these reagents do not affect the anti-apoptotic action of other anti-apoptotic genes, such as bcl-2. Exposure of EC to exogenous CO, under inhibition of HO-1 activity by SnPPIX, substitutes for HO-1 in preventing EC apoptosis.

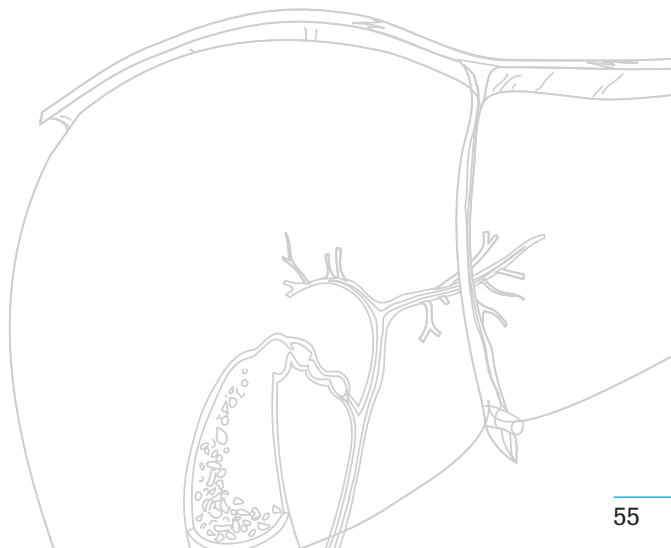
The mechanism of action of CO is dependent on the activation of the p38 mitogen-activated protein kinase (MAPK) signal transduction pathway (Brouard et al., 2000). Expression of HO-1 or exposure of EC to exogenous CO enhanced p38 MAPK activation by TNF- $\alpha$ . Specific inhibition of p38 MAPK activation by the pyridinyl imidazole SB203580 or by over-expression of a p38 MAPK dominant negative mutant abrogated the anti-apoptotic effect of HO-1. Taken together, these data demonstrate that the anti-apoptotic effect of HO-1 in EC is mediated by CO and more specifically via the activation of p38 MAPK by CO (Brouard et al., 2000). Given the key role of the transcription factor nuclear factor  $\kappa$ B (NF- $\kappa$ B) in regulating the expression of anti-apoptotic genes in EC (Soares et al., 1998), we questioned whether CO interacted with this signal transduction pathway to suppress EC apoptosis. Our data suggest that this is the case. These data are currently in the press and will be described in detail in the final ROTRF grant report.

We have previously defined the conditions that allow wild-type mouse hearts to accommodate (Koyamada et al., 1998). We will analyze *in vivo* whether the signaling pathways by which CO prevents EC apoptosis are directly involved in the induction of xenograft accommodation. To do so, we are testing whether hearts from mice genetically deficient in the upstream MAPK kinases that induce p38 MAPK activation, i.e. MKK3 and/or MKK6, can still undergo accommodation. The *mkk3*<sup>-/-</sup> and *mkk6*<sup>-/-</sup> mice used in these experiments were kindly provided by A.M.K. Choi and R. Flavell at Yale University. The results from these experiments will be described in detail in the final ROTRF grant report.

As suggested in the original grant proposal (specific aim 3), we are also analyzing the ability of HO-1 transgenic mouse hearts to undergo accommodation when transplanted into untreated, CVF- or CsA-treated rats. We have shown previously that normal mouse hearts transplanted under these conditions undergo acute vascular rejection in 4–5 days (Koyamada et al., 1998). The cardiac myocyte specific HO-1 transgenic mice used in these experiments were kindly provided by M. Perrella and S. F. Yet of the Brigham and Women's Hospital, Harvard Medical School. The results from these experiments will be described in detail in the final ROTRF grant report.

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**Dr. Stanislaw M. Stepkowski, Principle Investigator**

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*Dr. Robert A. Kirken, Consultant*



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### **Cytokine-Activated Signalling in T Cells is Required for Tolerance Induction by Allochimeric Protein**

The goal of our study is to understand the mechanism of transplantation tolerance induced by designed donor/recipient class I major histocompatibility complex (MHC) antigens. Our previous results showed that a single p.v. injection of 10  $\mu$ g  $\alpha_{1H}^{u70-77}$ -RT1.A<sup>a</sup> protein alone (bearing four donor polymorphic amino acids: His70, Val73, Asn74 and Asn77) induced tolerance to Wistar Furth (WF; RT1<sup>u</sup>) heart allografts in ACI (RT1<sup>a</sup>) recipients.

Our present study has shown that oral delivery of  $\alpha_{1H}^{u70-77}$ -RT1.A<sup>a</sup> protein (days 0–6) induces tolerance, as evidenced by inhibition of acute and chronic rejection. Delivery of  $\alpha_{1H}^{u70-77}$ -RT1.A<sup>a</sup> with therapeutic doses of cyclosporin (CsA) also prevented chronic rejection, which developed readily after treatment with CsA alone. Adoptive transfer experiments revealed that tolerance was mediated by potent regulatory T cells. Qualitative PCR-based analysis showed that tolerant recipients had reduced numbers of interleukin (IL)-2/interferon (IFN)- $\gamma$ -producing Th1 cells and elevated numbers of IL-4/IL-10-producing Th2 cells. The same T cells displayed diminished TCR-driven signaling via extracellular-regulated kinase, AP-1, and NF- $\kappa$ B, as well as the common  $\gamma$ -chain ( $\gamma_c$ ) cytokine-receptor-induced signaling by Janus kinase 3 (Jak3)/STAT5 pathways.

### **Hypothesis 1. Effective IL-4/STAT6-driven upregulation of Th2 is required for induction of tolerance by allochimeric protein**

During the first year of the grant funding we focused on generating alloreactive T cell clones specific to an immunogenic peptide (bearing nine polymorphic amino acids: 9u 62–77) by a standard limiting dilution method. We generated nine clones that proliferated in response to syngeneic antigen-presenting cell (APC) presentation

of the immunogenic 9u 62-77 peptide. One clone, LND-2, was characterized as CD4<sup>+</sup>, CD8<sup>-</sup>, TCR<sup>+</sup> and CD25<sup>+</sup> by FACS analysis with a panel of specific monoclonal antibodies (mAbs). To examine T cell receptor (TCR) signaling, the LND-2 clone was challenged with either immunogenic 9u 62-77 peptide or tolerogenic 4u 70-77 peptide (bearing four polymorphic amino acids: His70, Val73, Asn74 and Asn77), which are identical to those present in the tolerogenic  $\alpha_{1h}^{u70-77}$ -RT1.A<sup>a</sup> protein. The LND-2 T cell clone showed a potent proliferative response to 9u 62-77 peptide that was reduced by 70% when challenged with the tolerogenic 4u 70-77 peptide. Furthermore, the LND-2 clone did not respond to the immunogenic 9u 62-77 peptide unless presented by APC, nor to APC presenting the third-party 8u 62-82 peptide (bearing RT1.An polymorphic amino acids). Thus, the 9u 62-77 peptide-specific LND-2 T cell clone showed significantly reduced proliferative response to the tolerogenic 4u 70-77 peptide.

To examine the TCR-induced signaling pathway, the LND-2 T cell clone stimulated with 9u 62-77 or 4u 70-77 peptide was examined for phospho-Zap70 and total Zap70. Although the LND-2 clone stimulated with 9u 62-77 peptide showed maximal tyrosine-phosphorylated Zap70 at 10 min, the clone stimulated with 4u 70-77 peptide failed to produce detectable levels of phospho-Zap70. These results suggest partial TCR engagement of the LND-2 T cell clone by the tolerogenic 4u 70-77 peptide.

To examine the correlation between the partial TCR engagement and STAT5 activation, the LND-2 clone was stimulated with either 9u 62-77 or 4u 70-77 peptide for 4 days in the presence of 10 nM IL-2. Next, LND-2 cells were washed and cultured for 15 min with either 100 nM IL-2 or 100 nM IL-4. Nuclear extracts from each treated group were examined for binding to a <sup>32</sup>P-labeled STAT5-specific DNA probe by electrophoresis mobility shift assays (EMSA). The LND-2 clone stimulated with 9u 62-77 peptide showed potent STAT5 DNA binding induced by IL-2 but not by IL-4. The LND-2 clone stimulated with 4u 70-77 peptide displayed reduced STAT5 DNA binding following IL-2 stimulation and inducible STAT5 activation by IL-4. These results suggest that partial TCR engagement modifies the pattern of STAT5 activation by IL-2 or IL-4 in the LND-2 T cell clone. We plan to use the same clone (and other clones) to examine IL-4/STAT6 activation after stimulation with immunogenic and tolerogenic peptides.

## **Hypothesis 2: Delivery of a full second B7/CD28 signal is needed to induce tolerance by allochimeric protein**

To examine the role of signals 2 and 3 in induction of tolerance, ACI recipients of WF heart allografts received a single p.v. injection of 10  $\mu\text{g}$   $\alpha_{1\text{h}}^{\text{u70-77-RT1.A}^{\text{a}}}$  protein alone or in combination with a signal 1 inhibitor, CsA, a signal 2 inhibitor, CTLA-Ig, or a signal 3 inhibitor, either rapamycin or AG490. Induction of tolerance by administration of protein alone was not affected by the addition of CsA therapy. In contrast, concomitant application of the tolerogenic protocol with CTLA4-Ig, rapamycin (which blocks the mammalian target of rapamycin mTOR) or AG490 (which targets Jak3) prevented induction of tolerance. These results document that induction of tolerance by allochimeric protein requires both signals 2 and 3. These *in vivo* results were confirmed by the pattern of the STAT5 activation in T cells. In comparison with rejectors, cell extracts obtained on day 6 post-grafting from spleens of recipients treated with  $\alpha_{1\text{h}}^{\text{u70-77-RT1.A}^{\text{a}}}$  protein alone, CsA alone, or a combination of protein and CsA showed decreased levels of activated STAT5. Similarly, rapamycin alone and the rapamycin/ $\alpha_{1\text{h}}^{\text{u70-77-RT1.A}^{\text{a}}}$  protein combination displayed reduced activation of STAT5 because STAT5 is probably not dependent on the mTOR pathway. In contrast, since AG490 directly blocks the Jak3/STAT5 pathway, both AG490 alone and AG490/ $\alpha_{1\text{h}}^{\text{u70-77-RT1.A}^{\text{a}}}$  protein produced complete inhibition of STAT5 DNA binding activity. These results suggest that STAT5 may be required for induction of regulatory T cells. We intend to further examine STAT5 importance in transplantation tolerance.

## **Hypothesis 3: Apoptosis of alloantigen-specific T cells is not necessary for induction of tolerance by allochimeric protein**

We plan to perform *in vivo* experiments to examine whether alloantigen-specific T cells play a role in induction of tolerance by allochimeric proteins.

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## **Prof. Angus Thomson, Principle Investigator**

*Dr. Adrian Morelli, Co-Applicant*

*Dr. Peta O'Connell, Co-Applicant*

*Dr. Mohamed H. Sayegh, Consultant*

*Dr. Charles Maliszewski, Consultant*



## **University of Pittsburgh, Pittsburgh, USA**

### **Studies of a Novel Dendritic Cell (DC) Population in Organ Allograft Survival**

Dendritic cells (DC) are a heterogeneous population of rare leukocytes, uniquely specialized for antigen (Ag) presentation, that induce and regulate immune responses. Following organ transplantation, DC migrate from the periphery to draining secondary lymphoid tissue, where they present Ag to naive or resting T lymphocytes. Recent speculation that tolerance and immunity may be mediated by distinct DC subsets is buoyed by descriptions of phenotypically and functionally distinct DC populations in rodents and humans. CD11c<sup>+</sup> DC with the phenotype CD8 $\alpha$ <sup>+</sup> CD11b<sup>o</sup> or CD8 $\alpha$ <sup>-</sup> CD11b<sup>hi</sup> have been identified in and isolated from mouse lymphoid and non-lymphoid tissues, including bone marrow, thymus, blood, spleen, lymph node, Peyer's patches, lung and liver. Their relative incidences vary with tissue distribution: CD8 $\alpha$ <sup>-</sup> DC are the predominant subset in bone marrow and blood, while CD8 $\alpha$ <sup>+</sup> DC are the principal thymic DC. Adoptive transfer studies have demonstrated that CD8 $\alpha$ <sup>+</sup> and CD8 $\alpha$ <sup>-</sup> DC may both develop from highly purified, committed lymphoid and myeloid precursors. Thus, whether differential expression of CD8 $\alpha$  by DC accurately reflects developmental commitment to functionally distinct DC subsets, or the influence of microenvironmental factors, remains to be determined.

CD8 $\alpha$ <sup>+</sup> and CD8 $\alpha$ <sup>-</sup> DC reside in distinct microanatomic locations. CD8 $\alpha$ <sup>+</sup> DC localize in T cell areas of periarteriolar lymphocytic sheaths (PALS) in the spleen and lymph nodes. CD8 $\alpha$ <sup>-</sup> DC are found in marginal zones, but redistribute to the PALS following exposure to proinflammatory stimuli. In contrast to initial reports, we have shown that CD8 $\alpha$ <sup>+</sup> DC migrate from peripheral subcutaneous sites to draining lymph nodes. They also traffic to the spleen following intravenous administration. CD8 $\alpha$ <sup>+</sup> DC are major producers of IL-12p70 and IFN- $\alpha$  compared with CD8 $\alpha$ <sup>-</sup> DC, and are the only DC subset to cross-prime CD8<sup>+</sup> T cells *in vivo*.

Although both CD8 $\alpha^+$  and CD8 $\alpha^-$  DC stimulate T cell responses efficiently, CD8 $\alpha^+$  DC can also regulate T cell proliferation *in vitro*. Compared with their CD8 $\alpha^-$  counterparts, CD8 $\alpha^+$  DC induce elevated levels of CD95 (Fas)-CD95L-dependent apoptosis of CD4 $^+$  T cells, and restrict CD8 $^+$  T-cell proliferation by limiting their ability to produce IL-2. Although Ag (keyhole limpet hemocyanin, or ovalbumin)-pulsed CD8 $\alpha^+$  and CD8 $\alpha^-$  DC administered subcutaneously prime Ag-specific CD4 $^+$  T cells with equivalent efficiency, the former induce predominantly Th1 responses, whereas CD8 $\alpha^-$  DC drive Th2 or mixed Th1/Th2 responses.

Recent evidence has strengthened the view that either donor or host DC, particularly those that are immature, can regulate anti-donor reactivity and prolong graft survival. The majority of studies that have investigated the function and potential therapeutic utility of DC in allo- or auto-immunity, have used either bulk DC isolated directly from tissues, or myeloid DC generated *in vitro* using GM-CSF ( $\pm$  IL-4). With the support of the ROTRF, we have examined the influence of CD8 $\alpha^+$  DC mobilized in donor-strain animals in response to Flt3 ligand and administered prior to transplant on anti-donor immune reactivity and organ allograft survival. We have observed that both immature and mature CD8 $\alpha^+$  DC, but only immature CD8 $\alpha^-$  DC significantly prolong heart transplant survival in the absence of anti-rejection therapy. Conditioning with CD8 $\alpha^+$  DC was not accompanied by evidence of T-cell deletion or immune deviation at the time of transplant. However, within 5 days of transplantation, donor-specific T-cell responses were significantly impaired in mature CD8 $\alpha^+$  DC-treated mice. These novel observations provide evidence of an *in vivo* immunoregulatory activity of CD8 $\alpha^+$  DC in the context of alloimmunity.

In addition to efficiently mobilizing CD8 $\alpha^-$  and CD8 $\alpha^+$  DC subsets in mice, Flt3 ligand dramatically increases DC1 and DC2 precursors in humans. In contrast, G-CSF augments only DC2 precursors, which are the putative homologues of murine CD8 $\alpha^+$  DC. As shown in our study, these DC are capable of prolonging allograft survival in the absence of immunosuppression, and irrespective of their maturational status. Conceivably, differential mobilization and isolation of such a regulatory DC subset from prospective organ donors may allow their evaluation in tolerance-enhancing strategies in clinical transplantation. In the third year of the project, we propose to further evaluate the therapeutic potential of the CD8 $\alpha^+$  DC subset and the mechanism underlying their activity.

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O'Connell PJ, Logar AJ, Truneh A, Thomson AW. Differential regulation of allogeneic T cell proliferation and apoptosis by immature hepatic dendritic cell subsets. *Am J Transplant* 2001; 1: S449.



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## Gene Expression of Tolerance-Mediating Allospecific Cells

### Introduction

Recently, we showed that the non-depleting anti-rat CD4 mAb RIB5/2 is a powerful inducer of tolerance to allografts. The tolerance is adoptively transferable by spleen or graft-infiltrating cells in an infectious manner. In order to characterise these cells in more detail, we performed differential display RT-PCR and suppression subtractive hybridisation with graft-infiltrating cells from rat allograft recipients treated with anti-CD4 and control mAb. We defined several gene fragments that were specifically up- or down-regulated in graft-infiltrating cells from rats treated with anti-CD4 mAb. Full-length cDNA of one of these fragments showed high homology to the anti-apoptotic gene Bag-1, first described in the mouse. *In vitro* allostimulation in the presence of anti-CD4 mAb strongly up-regulates Bag-1 expression in responder T cells. The associated relative resistance to apoptosis may explain the long-lasting survival of regulatory T cells *in vivo* despite persistence of alloantigen.

The present project focuses on the following topics.

- 1) Bag-1.** In order to prove the hypothesis that Bag-1 is essential for stable tolerance, we will study whether Bag-1 knockout mice (which are under development) are able to develop tolerance-mediating T cells. Additionally, we will determine whether *ex vivo* down-regulation of Bag-1 by antisense technology in T cells derived from rats treated with anti-CD4 mAb abolishes their tolerance-mediating properties.
- 2) New genes.** We will further characterise other gene fragments specifically up- or down-regulated in our system. We have concentrated our work on expression kinetics studies of all isolated fragments in various transplantation tolerance models using real-time PCR in order to identify some reliable tolerance-associated markers for post-transplant monitoring.

**Specific Aim 1. Investigation of the importance of anti-apoptotic proteins (e.g. Bag-1) for the generation of long-living regulatory T cells and the maintenance of allograft tolerance**

*Blocking Bag-1 protein expression in vivo by applying antisense oligonucleotides.* In the last report (April 2000), we showed that inhibition of Bag-1 expression *in vitro* leads to increased sensibility against spontaneous and activation-induced apoptosis of the cells derived from an anti-CD4-mAb-treated allogeneic mixed lymphocyte reaction. We are currently trying to inhibit Bag-1 expression in tolerance-mediating T cells from kidney and heart recipients treated with anti-CD4 mAb before adoptive transfer. These experiments may show whether an enhanced Bag-1 expression is needed for the induction and maintenance of transplantation tolerance.

*Generation of Bag-1 knockout mice.* We isolated and sequenced the genomic clone of the mouse Bag-1 gene. The complete genomic clone consists of 9708 bp and contains 7 exons. We have chosen exon 2 as a target to generate a T-cell-specific knockout mouse. This work will be carried out within the next year.

*Expression studies of other apoptosis-associated proteins (e.g. Bcl-2) in vitro and in vivo.* In the last report we demonstrated that enhanced protection against apoptosis of cultures treated with anti-CD4 mAb is not due to differences in Bcl-xl and Bax expression between treated and non-treated allogeneic cultures or to diminished FasL expression after restimulation of responder T cells. Currently, we are analysing the expression of different anti- and pro-apoptotic proteins *in vivo* after transplantation (see also below).

**Specific Aim 2. Characterisation of other genes differentially expressed in the renal transplantation model**

*Further studies of the cDNA fragment ACD4-2, which shows strong homology to the phosphatidylinositol transfer protein (PITP).* We generated a primer/probe panel for use in real-time PCR. This panel will be used to analyse the expression of PITP during mixed lymphocyte reactions comparing anti-CD4-mAb-treated and non-treated allogeneic cultures. Furthermore it will be used to investigate its expression at different time points in several transplantation models (see also below).

**Specific Aim 3. Expression studies in other transplantation models**

We have recruited samples from several transplantation tolerance models that will



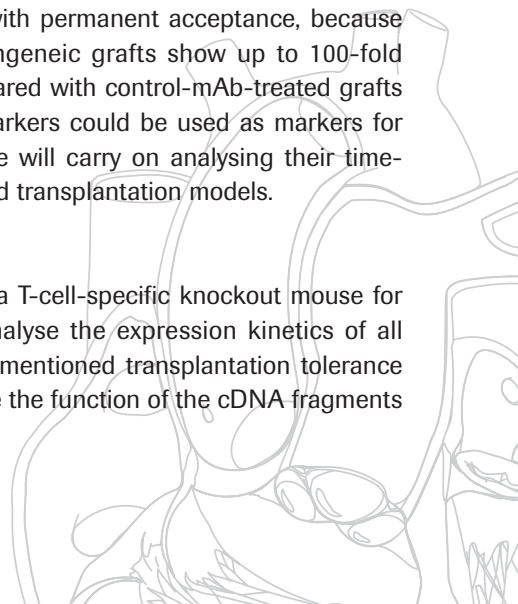
be used to analyse the expression kinetics of all isolated cDNA fragments. First we used the non-depleting anti-CD4 mAb RIB5/2 (10 x 20 mg/kg body weight) to induce donor-specific tolerance against renal allografts into different strain combinations: Wistar Furth to BDIX; and DA to Lewis. The expression of the isolated cDNA fragments will be analysed at days 2, 5, 10, 14, 25 and >100 and compared with the expression at days 2 and 5 of non-treated control animals. The same expression studies will be carried out using heart allografts. Furthermore the expression of the isolated cDNA fragments will be analysed after CTLA4-Ig treatment in renal and heart allografts.

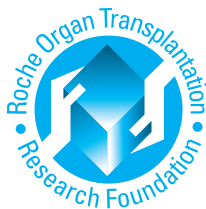
The “adoptive tolerance” model was established by Tullius et. al. A Fisher allograft is transplanted into Lew recipients, leaving one of its own kidneys inside. The allograft undergoes chronic rejection. Four weeks later both kidneys will be removed and a second allograft transplanted. This second renal allograft will be permanently accepted without any treatment. Samples from both the first allograft (as control) and the second allograft 2, 4, 8, 12 and 16 weeks after transplantation will be analysed for expression of the isolated cDNA fragments. At the moment we are recruiting samples from two mouse tolerance models that will be used to compare the expression kinetics between different species.

We have generated primer panels for all isolated cDNA fragments, which will be used to quantify their expression. So far we have analysed the time-dependent expression pattern of some isolated fragments in the first described model (kidney, Wistar Furth to BDIX). Especially the fragments DDRT-T4, DDRT-T5, DDRT-T8 and DDRT-T10 show a strong association with permanent acceptance, because anti-CD4-treated allogeneic grafts and syngeneic grafts show up to 100-fold higher expression of these fragments compared with control-mAb-treated grafts at the time of rejection ( $\approx$  day 5). These markers could be used as markers for post-transplant monitoring. To verify this we will carry on analysing their time-dependent expression in the other mentioned transplantation models.

### Future plans

In the next year we would like to generate a T-cell-specific knockout mouse for Bag-1. Furthermore we will continue to analyse the expression kinetics of all the isolated cDNA fragments in the above-mentioned transplantation tolerance models. We will also continue to characterise the function of the cDNA fragments ACD4-3 and ALLO-5.





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